CYTOLOGY OF THE BLOOD AND BONE MARROW OF GUINEA-PIGS FOLLOWING HAEMORRHAGE, AND ADMINISTRATION OF LIVER EXTRACT

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Considering the extensive use of the guinea-pig in routine and research laboratory procedures it is surprising to find little mention of haematological data regarding this animal in the literature. Articles by King *et al.* (1941), Jacobsen (1934) and Pattison and McDiarmid (1943) show that there is considerable variation in peripheral blood standards and that such variation is dependent upon dietary conditions. Bone marrow examinations were not recorded by any of the authors. The above fact was utilized by Jacobsen (1934) (1935) and Gall (1937) who regarded the normal marrow as being in a state of permanent megaloblastosis comparable to that seen in the bone marrow of cases of human pernicious anaemia. The authors nominated the guinea-pig as a suitable experimental animal for the assay of anti-pernicious anaemia extracts owing to the occurrence of a transient reticulocytosis following administration of such principles. Contrary to this statement is the work of Jones (1936) who regarded the promegaloblasts and megaloblasts of Jacobsen and Gall as being normal precursors of the red cell series, i.e. proerythroblasts (pronormoblasts) and normoblasts.

From the foregoing review it will be seen that quite apart from any absolute figures as to the number of cells present, there is a discrepancy as to the morphology of the cellular elements themselves. It is not proposed to enter a discussion on a subject of such a controversial nature but to use the published facts as a basis for the investigation outlined below.

METHODS.

From a preliminary survey of both blood and bone marrow it was obvious that so-called "normal" guinea-pigs could be divided into two groups according to whether their diet was enriched by bran or not. Animals supplied with carrots, cabbage, straw and water, with the addition of bran stopped six weeks prior to examination, constituted group A. Those of group B had bran continuously included in the above diet. Neither group had had previous administration of liver extract, and to ensure that uniform conditions prevailed each animal was confined to one cage and all housed in the same room.

Serial blood counts were made on oxalated blood drawn by cardiac puncture. Bone marrow was obtained by section and aspiration of the femur. Films of blood and marrow were stained by both Graham's peroxidase method and Leishman's stain. Material for histological preparations was fixed in Zenker's fluid and stained by haematoxylin and eosin (Weigert).

For the production of haemorrhage 3·0 ml. blood were withdrawn by cardiac puncture after the normal limits of blood variation had been computed. The anti-pernicious anaemia extract was that manufactured by British Drug Houses under the name of "Anahaemin"; this was administered in 0·25 ml. amounts by intramuscular inoculation.

In the Tables the results are expressed as follows.

Red blood count: R.B.C. in millions per c.mm.
Haemoglobin: Hb. gm. per 100 ml. blood.
Mean corpuscular haemoglobin: M.C.H. in micromicrograms.
Reticulocytes: Retic. per cent.

In the differential count the cells are recorded as percentages. The nomenclature in the case of bone marrow elements is a combination of that proposed by Dameshek and Valentine (1937) and Israels (1940) in which all nucleated red cells are regarded as erythroblasts and classified as normoblasts according to maturation stages by the letters A, B and C.

EXPERIMENTAL RESULTS.

Animals of group A showed wide variation in peripheral cellularity. A series of thirty guinea-pigs gave red cell counts varying from 2,800,000 to 4,400,000 per c.mm. with haemoglobin ranges of 7·8 to 11·9 gm. per 100 ml. blood. Differential counts showed great variation in

leucocytes which commenced in most animals as a relative lymphocytosis but could be completely reversed to a polymorphonucleocytosis over the examination period of one week. Daily examination of two such animals (Fig. 1) showed that apart from variations which could be attributed to slight loss of blood following removal of the specimens, the observed figures were accounted for by the normal daily fluctuation in red cells.

TABLE 1.

Pronormoblasts.	Normoblasts A.	Normoblasts B.	Normoblasts C.	Myeloblasts.	Promyelocytes.	Neutrophil Myelocytes.	Eosinophil Myelocytes.	Metamyelocytes.	"Stabs."	"Segmentals."	Eosinophils.	Basophils.	Lymphocytes.	Monocytes.	Kurloff cells.	Sex.	Weight (ozs.).
—	0·3	4·6	22·4	1·7	0·4	0·3	0·5	4·2	11·5	23·6	7·6	1·0	21·0	0·6	0·3	F	12
—	—	2·0	15·0	1·5	0·5	1·5	0·5	6·5	10·5	32·0	6·5	1·5	21·0	1·0	—	M	10
—	—	5·0	19·0	1·8	0·8	1·0	—	2·4	13·7	33·0	5·3	0·2	24·8	1·0	—	M	12
—	0·2	3·8	23·1	1·9	0·5	0·4	0·3	3·8	11·8	26·0	5·1	0·8	21·6	0·9	—	F	8
—	0·4	3·3	22·0	1·5	1·0	1·0	1·0	2·5	11·0	27·8	8·5	—	17·0	1·0	1·0	F	12
—	—	0·5	21·5	1·5	0·5	—	—	2·5	8·0	38·0	0·5	1·5	24·0	0·5	1·0	F	10
—	0·5	3·0	21·4	1·4	0·5	0·5	1·5	3·0	10·5	28·5	6·4	0·6	19·2	0·5	2·5	F	14
—	0·4	3·2	22·1	1·2	0·9	0·8	1·3	2·8	10·5	28·4	7·6	0·5	17·4	2·0	—	M	12
0·5	0·5	4·8	21·5	1·5	1·0	0·5	1·5	2·5	9·0	30·0	4·0	—	22·4	1·0	0·5	F	10
—	0·3	3·8	19·8	1·9	0·8	1·0	1·2	3·8	10·8	24·6	6·9	1·5	22·0	1·0	—	F	12
—	0·4	3·1	21·5	1·2	0·7	0·8	1·2	2·4	11·1	29·5	5·4	—	20·8	1·2	1·8	M	10
—	0·5	3·2	19·8	1·2	0·2	0·8	1·2	2·7	11·3	28·1	6·8	1·0	18·6	1·4	0·6	F	10
—	0·3	3·0	22·1	1·9	0·4	0·9	0·8	2·8	13·3	26·0	6·2	1·4	20·0	1·0	—	M	13
—	0·5	7·0	28·5	0·5	0·5	1·5	0·5	2·5	9·5	27·0	1·5	1·5	17·0	0·5	1·5	F	10
—	0·5	8·0	34·0	0·5	0·5	2·0	—	3·0	3·5	30·5	0·5	—	16·0	0·5	—	F	8
—	0·4	8·3	33·8	0·5	0·5	1·3	0·3	2·8	4·0	30·6	0·8	0·3	16·3	—	0·2	M	12
—	0·5	8·6	32·4	0·2	0·2	1·6	0·2	1·8	8·4	29·1	0·6	0·1	16·2	0·1	—	M	12

Correlation of the above counts with the cytology of the bone marrow shows that the latter is in a state of hyperplasia. This tissue consists of dense masses of packed cells of both erythroblastic and granulocytic series (together with their precursors) supported by a framework of reticulated fibres and elongated fibroblastic cells. Fat globules are reduced in number. In all marrows small aggregations of round lymphoid cells were seen in the vicinity of the nutrient artery. These cells were unidentifiable in histological sections but shown to be small lymphocytes in smear preparations. Differential counts from such smears are given in Table 1; each count represents one animal.

In the second group of animals (with bran enriched diet) there was less erythropoietic activity with an increase in the granulocytic series. Blood counts from these animals showed more or less uniform figures although the variation seen in differential counts of leucocytes in the previous class of test animals still persist. (R.B.C. variation 3,780,000 to 4,200,000; Hb. 10·2 to 11·1 gm. per 100 ml.).

Bone marrow examinations (Table 2) show a decrease in erythroblasts and an increase in "stab" and "segmental" forms of neutrophil leucocytes. There was no reduction in cellularity over the previous class of animals. Apparently the increase in granulocytic elements is related to the lowered activity of the red cell series. It must therefore be concluded that hyperplasia is still the dominant factor in conjunction with a maturation effect induced by the addition of cereal to the diet.

TABLE 2.

Pronormoblasts.	Normoblasts A.	Normoblasts B.	Normoblasts C.	Myeloblasts.	Promyelocytes.	Neutrophil Myelocytes.	Eosinophil Myelocytes.	Metamyelocytes.	"Stabs."	"Segmentals."	Eosinophils.	Basophils.	Lymphocytes.	Monocytes.	Kurloff cells.	Weight (ozs.).	Sex.
—	0·2	2·0	14·0	1·2	0·4	0·8	1·2	2·8	7·0	40·9	4·6	1·2	22·9	1·2	0·2	19	M
—	0·1	1·8	11·8	0·8	0·3	0·6	0·8	1·9	10·0	45·4	6·2	—	19·2	0·8	—	10	F
—	—	2·2	12·6	2·0	0·6	0·4	0·4	1·8	8·0	45·3	4·2	1·0	19·9	0·6	—	8	M
—	0·1	0·9	11·6	2·3	—	1·0	—	2·4	14·2	38·3	3·8	—	26·8	—	0·6	12	M
—	0·3	1·6	12·9	1·0	1·2	2·4	0·8	2·6	18·2	33·3	2·0	0·8	22·6	0·1	0·2	14	M
—	0·2	1·0	10·2	2·3	0·2	0·8	0·2	1·6	17·2	38·3	3·4	0·3	24·3	—	—	12	F

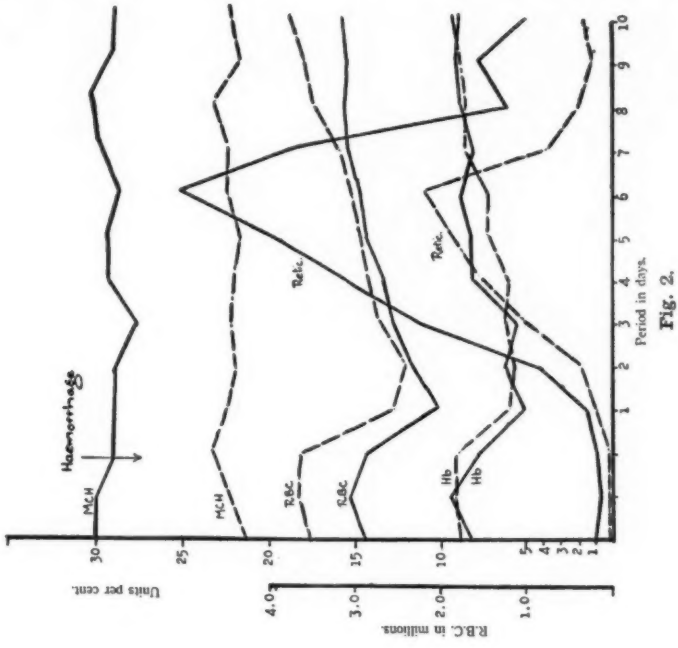
Haemorrhage
MCH
MCH
RBC
RBC
Retic.
Retic.
Hb
Hb
Units per cent.
R.B.C. in millions.
Period in days.

Fig. 2.

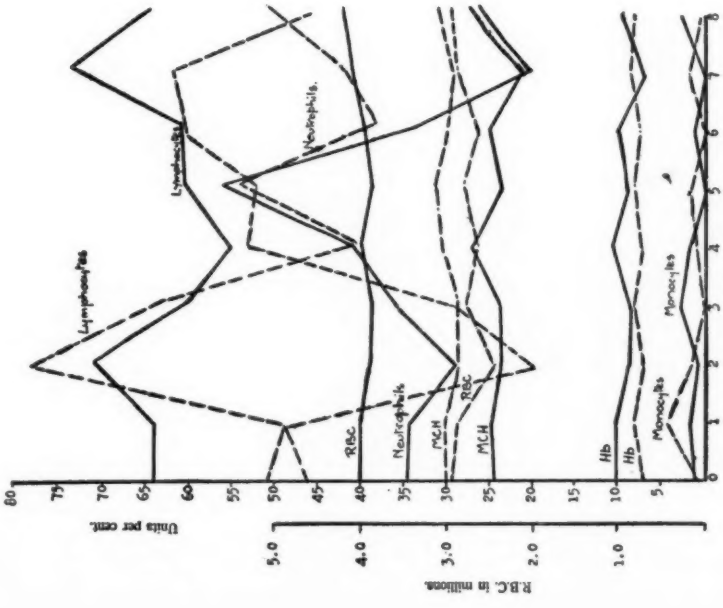
Lymphocytes
Lymphocytes
Neutrophils
Neutrophils
RBC
RBC
MCH
MCH
Hb
Hb
Monocytes
Monocytes
Units per cent.
R.B.C. in millions.
Period in days.

Fig. 1.

The post-haemorrhagic blood changes are naturally influenced by the initial red cell level. The following results were obtained from animals on a bran enriched diet. Immediately following blood loss there is an abrupt drop in the total red cell count with no alteration in the mean corpuscular haemoglobin. Within the next few days a slow rise in red cells and haemoglobin occurs which increases in magnitude as marrow stimulation takes place. Reticulocytes increase to a maximum on the sixth or seventh day. The extent of this crisis is proportional to the stimulus applied and to the pre-existing level of the blood (Fig. 2). It would appear that the higher reticulocyte crisis in the case of the animal with the lower blood level is probably due to the bone marrow being in a more active state, probably caused by semi-anaemic conditions.

Bone marrow examinations over a period of eight days following blood loss show that the peak of erythropoietic activity coincides with the reticulocyte crisis. The correlation between peripheral blood and bone marrow findings is shown in Tables 3 and 4.

TABLE 3.

	Animal 412. Fem. 12 ozs.				Animal 408. Fem. 10 ozs.				Animal 409. Fem. 12 ozs.			
Days.	RBC.	Hb.	MCH.	Retic.	RBC.	Hb.	MCH.	Retic.	RBC.	Hb.	MCH.	Retic.
—	4·3	9·4	21·9	0·1	3·64	8·1	22·0	0·1	4·10	10·2	24·9	1·0
*	→4·26	9·4	22·0	0·1	→3·72	8·7	23·4	0·1	→4·16	10·2	24·0	0·8
1	2·83	8·7	30·7	0·3	2·00	5·4	27·0	1·6	2·30	6·3	27·3	1·5
2	2·96	7·8	27·0	2·6	2·26	5·4	23·9	8·2	2·91	7·1	24·8	2·6
3	3·26	7·1	21·7	3·9	2·62	6·3	24·0	9·8	3·00	7·1	23·6	3·4
4	3·42	8·2	23·9	6·8	2·88	7·1	24·6	12·8	3·16	7·8	21·7	4·8
5	3·62	9·4	26·0	10·2	2·96	8·0	29·0	12·6	Animal killed.			
6	3·81	9·4	24·6	12·3	Animal killed.							
7	3·82	9·0	23·5	12·2								
	Animal killed.											

* At the arrow 3 ml. of blood withdrawn.

TABLE 4.

Pronormoblasts.	Normoblasts A.	Normoblasts B.	Normoblasts C.	Myeloblasts.	Promyelocytes.	Neutrophil Myelocytes.	Eosinophil Myelocytes.	Metamyelocytes.	"Stabs."	"Segmentals."	Eosinophils.	Basophils.	Lymphocytes.	Monocytes.	Kurloff cells.	Animal No.
—	0·3	4·9	36·2	0·6	0·2	—	0·5	0·3	6·3	23·8	4·9	—	21·8	0·2	—	409
0·8	2·8	7·2	41·2	0·3	—	1·2	0·2	0·6	3·8	14·4	1·2	—	23·0	0·3	1·0	408
0·7	3·3	38·6	39·8	0·5	0·3	—	—	0·3	5·0	5·6	0·6	0·3	5·0	—	—	412
1·0	4·6	6·3	56·0	1·2	1·2	1·4	0·2	1·0	3·0	8·0	0·8	0·2	5·6	—	0·1	6
1·0	1·2	22·4	47·2	0·2	0·2	0·6	—	1·0	5·0	11·2	0·8	—	5·8	1·4	0·2	7
—	0·6	18·0	55·2	0·6	0·6	1·0	—	1·0	2·6	10·2	0·6	1·0	5·0	0·3	—	9
1·3	6·0	28·0	46·0	1·0	1·0	—	—	—	3·3	8·6	—	—	4·0	—	1·0	13
1·6	0·9	26·1	48·5	0·2	0·2	0·8	0·1	0·8	3·8	10·2	0·8	—	5·8	0·3	—	28
0·8	4·6	29·8	46·0	0·8	0·8	1·0	—	1·0	4·0	6·6	0·8	—	3·6	—	0·6	16
0·4	3·8	26·8	50·3	0·4	0·4	0·4	0·3	0·7	2·4	6·3	0·7	0·3	6·8	—	—	14

Bone marrow examination of guinea-pig No. 409 sacrificed on the fourth day after blood loss and whose reticulocytes had not yet reached their expected maximum showed moderate erythropoietic stimulation as evidenced by Type B and C normoblastic proliferation. Animal No. 408 was killed on the fifth day just prior to the expected reticulocyte crisis and showed a hyperplastic marrow with numerous mitotic figures. Erythroblasts replaced myeloid and lymphoid elements and appeared as dense masses of darkly-staining nuclei with a thin peripheral strip of cytoplasm (Type B normoblasts). Guinea-pig No. 412 was killed at the reticulocyte crisis and many mitotic figures in the erythroblast series testified to the great activity of these cells. Macroscopically the marrow was cherry red in colour and easily removed from the medullary cavity. Histological preparations showed the whole marrow cavity to be filled with small intensely staining round cells, several large pronormoblasts and an occasional fibroblast together with reduced numbers of cells of the granulocytic series. Fat cells were absent. Bone spicules were comparatively rare owing to their decalcification and absorption to increase the central cavity to meet with the added demands on the marrow by haemorrhage. Both normal and "bled" animals were treated with "Anahaemin" (0·25 ml. ml. intramuscularly). Fig. 3 is representative of the first class of animal. After blood loss the effect of liver on the reticulocyte level is masked by the natural post-haemorrhagic reticulocytosis, but if administration is delayed until after the crisis has been reached a second response of greater magnitude is realized (Fig. 4). The second

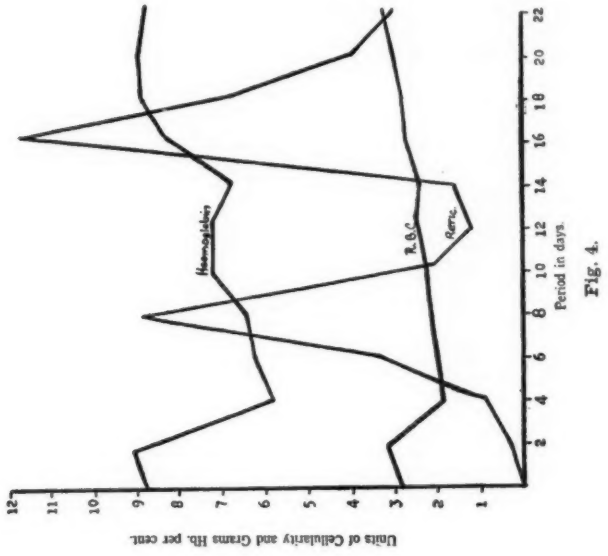
Units of Cellularity and Grams Hb. per cent.
Hæmoglobin
R.B.C
Reticulocytes
Period in days.

Fig. 4.

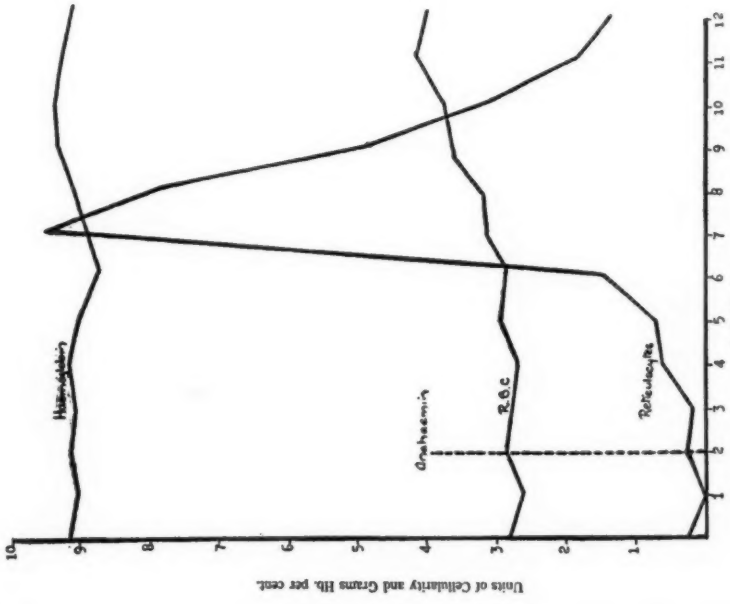
Units of Cellularity and Grams Hb. per cent.
Hæmoglobin
Anhæmia
R.B.C
Reticulocytes
Period in days.

Fig. 3.

reticulocytosis is of shorter duration and the following erythrocyte increase is relatively slight.

The bone marrow (Table 5) shows an increase in fat vacuoles and maturation of erythroblastic elements. Hypoplasia of all series is present with an increase in fibrous tissue. Erythroblastic activity is confined to normoblasts Type B and C, the general appearance of which indicates advanced maturation.

TABLE 5.

Pronormoblasts.	Normoblasts A.	Normoblasts B.	Normoblasts C.	Myeloblasts.	Promyelocytes.	Neutrophil Myelocytes.	Eosinophil Myelocytes.	Metamyelocytes.	"Stabs."	"Segmentals."	Eosinophils.	Basophils.	Lymphocytes.	Monocytes.	Kurloff cells.	Weight (ozs.).	Days after Hae.
—	—	5·0	19·0	1·8	0·8	1·0	—	2·4	13·7	33·0	5·3	0·2	24·8	1·0	—	8	2
—	—	3·5	21·5	1·5	1·5	3·5	0·5	3·0	6·5	30·0	2·5	0·5	25·0	—	2·5	16	3
—	—	3·6	26·4	1·5	1·5	2·1	1·6	3·4	4·6	40·1	4·1	0·5	10·2	0·3	0·2	10	4
—	0·5	8·0	34·0	0·4	0·4	2·0	—	3·0	3·6	30·4	0·5	0·1	15·9	0·5	—	12	5
0·4	0·6	4·8	21·4	1·6	1·6	0·5	1·5	2·5	9·0	30·0	4·0	—	22·0	1·2	0·3	12	6
—	0·6	18·1	55·2	0·5	0·5	1·0	—	1·0	2·6	10·9	0·6	1·2	4·8	0·3	—	10	7
—	0·2	2·1	13·9	1·1	1·1	0·7	1·3	2·8	7·1	40·8	4·6	1·2	22·9	1·3	0·1	10	8

In no animal, either before or after administration of liver extract were cells morphologically identical with any types of the human megaloblast seen in the bone marrow. Apparently the marrow of the guinea-pig is in a normoblastic state of erythroblastic activity with cessation of maturation at the Type B and C level. On the administration of the factor contained in liver extract necessary for this process to continue the numbers of these cells show a transient increase on the fourth to sixth day and thereafter a gradual decrease in numbers with evidence of stimulated erythropoiesis. The nature of this response to liver is of a normoblastic variety and the peripheral blood can be maintained at the higher level by continued administration of the maturation factor.

SUMMARY.

Several factors pertaining to the haematological standards of the guinea-pig under dietary and haemorrhagic conditions have been investigated.

The blood count of the guinea-pig under normal conditions shows slight individual daily fluctuations from a constant figure. Greater variation is shown in a comparison of counts of one animal with another.

Bone marrow examinations show a variation in erythropoietic activity according to the diet provided for the animal.

Post-haemorrhagic conditions show a pronounced reticulocytosis on the fourth to sixth day with marked hyperplasia and erosion of the medullary cavity of the long bones.

Administration of liver extract elicits a transient reticulocytosis accompanied by a normoblastic and not megaloblastic bone marrow. These results are in agreement with those obtained by Jones (1936) and contrary to the work of Jacobsen (1934) and Gall (1937).

Owing to the small number of animals used in the experimental work the conclusions arrived at must be interpreted with some caution.

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ON CRUORALBIN AND ITS PROSTHETIC GROUP

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The name "cruoralbin" was provisionally assigned to a green substance formed by the alternate reduction and reoxidation of oxyhaemoglobin in the presence of a high concentration of hydrogen cyanide (Holden, 1943b). The present paper records additional facts about it and describes the preparation and some properties of its prosthetic group, "cruoratin."

The preparation of cruoralbin has followed essentially the method previously described using a mixture of 25 ml. washed horse erythrocytes laked with 25 ml. water, 50 ml. 10 p.c. ammonium chloride and 50 ml. freshly prepared 10 p.c. potassium cyanide. The mixture was kept at about 15–18° C. for a few hours till the oxyhaemoglobin had been changed into cyan-methaemoglobin. During the subsequent preparation of cruoralbin the bottle was rotated in a box containing broken ice as it was observed that only from cruoralbin prepared at a low temperature could the cruoratin be subsequently detached in more than minute quantities. The sodium hyposulphite was added frequently and in small quantities to avoid as far as possible oxidation of all the protohaematin pigment present or of much excess of sodium hyposulphite. Conditions favouring the formation of hydrogen peroxide, i.e. excess of reducer and oxygen were found to impair the product. Finally the cruoralbin was oxidized by air. When the cruoralbin was to be studied as such it was precipitated with ammonium sulphate (120 gm.), filtered off and washed with a little saturated ammonium sulphate. The precipitate was redissolved in a little water, the stroma and denatured protein removed by centrifuging and the ammonium sulphate dialysed away overnight. All these processes were carried out as far as possible at 0° C. Next day the solution was filtered through a pleated paper before use.

The iron analyses were performed as follows: A suitable, accurately measured volume of solution was evaporated to dryness in a quartz test tube in a vacuum desiccator. It was ashed with 0·5 ml. sulphuric acid and the minimal amount of perchloric acid, added drop by drop. To avoid loss by spray when the latter was added the mouths of the quartz tubes were closed with small pyrex funnels. After ashing and cooling 2 ml. of water were added and the tubes kept in an oven at 40° C. over night to hydrolyse any iron pyrophosphate or other unionized iron compound present. This precaution was found both essential and adequate. The part of the valuable critical paper by Jackson (1938) dealing with this matter can be confirmed. Next day the solution in each tube was made up with distilled water to 10·0 ml. in a standard flask. To 1·0 ml. of this solution was added either 1·0 ml. or 2·0 ml. of $\alpha\alpha'$ dipyridyl in concentrated ammonium acetate solution. After careful mixing enough solid sodium hyposulphite was added to reduce the iron to the ferrous state. The right amount of dipyridyl was determined by a small scale trial.

The final determination was made with a visual colorimeter fitted with micro-cups. The standard iron solution used for comparison contained an appropriate amount of sulphuric acid to balance that used in the ashing. Conditions were so chosen that the concentration of iron in the unknown solution approached that in the standard solution. All reagents were purified as carefully as possible, the dipyridyl by distillation in steam, the water by distillation in a pyrex still with a special spray tap and the acids *in vacuo* in an all-glass still with a Claisen head. For the determination of inorganic iron in the cruoralbin solutions the protein was precipitated with purified trichloracetic acid and centrifuged off.

The so-called "labile iron" was liberated as usual by heating over night at 37° C. in the presence of 0·1 N hydrochloric acid. It was in general about 8–11 p.c. of the total iron. The amount of labile iron depends greatly on the proportion between the protein and the acid. Its value as a means of characterizing substances does not appear to be very high. Protein determinations were made by heat coagulation in the presence of formalin and weighing the washed and dried coagulum.

For the carbon monoxide determinations the spiral tonometer was used as for the determination of carbon monoxide in blood (Holden, 1943a). While less exact than some other methods it has two advantages which were helpful at this stage of the work: it is specific and it can be used on a small quantity of a relatively weak solution.

[1] This work was aided by a grant from the Australian National Health and Medical Research Council.

The ultra-violet absorption spectra were determined with a Hilger medium spectrograph and échélon cells. For the visible spectrophotometric curve a photo-electric spectrophotometer was used.

TABLE 1.

The iron content of cruoralbin and its ability to combine with carbon monoxide; calculations are based on the assumption that the equivalent weight of cruoralbin is about the same as that of haemoglobin and that one atom of iron combines with one molecule of carbon monoxide.

Substance.	Iron / Protein.	CO / Iron.
Haemoglobin	0·96	1·0
Cruoralbin	0·92	—
Cruoralbin	0·87	1·0
Cruoralbin	0·80	0·9

THE PREPARATION OF CRUORATIN.

During the preparation of cruoralbin only a small part of the pigment appears to be degraded to an iron-free type. Many attempts were made to prepare bile pigments from freshly made cruoralbin by those methods which have been so successful in the case of choleglobin (Lemberg, 1941). The yields were, however, quite insignificant. It was, however, observed that an old specimen which had been stored at 0° C. yielded a small amount of pigment into acetic acid and ether. This included some of the pigment whose reduced form showed a strong absorption band in the red portion of the spectrum.

The only reagent so far found that would satisfactorily detach the green prosthetic group from fresh cruoralbin is amyl alcohol. While it is soluble in other organic solvents, such as ether, chloroform, and ethyl acetate, they cannot dissolve it away from the protein of cruoralbin at any pH at which the prosthetic group is stable. As indicated above, if cruoralbin be prepared at 18–20° C. the proportion of cruoratin which can be detached appears small but the yield is increased if the preparation of the cruoralbin be carried out with the bottle of cyan-methaemoglobin previously cooled to 0° C. and surrounded by ice in an insulating box during the reductions and reoxidations.

Cruoratin is unstable and solutions have had to be prepared and used at once. In spite of numerous attempts by different methods no satisfactory solid specimen has been made and its complete separation from haematin effected only at the cost of great losses and with the disadvantage of finishing with a weak solution. A solution suitable for certain experiments was made as follows: Instead of precipitating the fresh cruoralbin with ammonium sulphate it is placed in the cold room at 0° C. and the subsequent steps done there. In this order are added to it, 100 ml. glacial acetic acid, 250 ml. redistilled fermentation amyl alcohol and, after mixing, 400 ml. ice cold water. The bottle is then briskly shaken for a couple of minutes and left standing for half an hour. The lower aqueous layer is sucked off and discarded. The protein is filtered off on a 15 cm. Buchner funnel. The filtrate containing the pigment is placed in a 1 litre separating funnel. The protein is washed with 200 ml. amyl alcohol containing about 20 ml. glacial acetic acid and again filtered, after which it is discarded. The second filtrate is added to the first in the funnel. The united filtrates are washed four times with 500 ml. water, sufficient ethanol being added, when necessary, to promote a reasonable separation of the phases. The aqueous layers are discarded. The amyl alcohol layer is filtered through a 9 cm. Buchner funnel and placed in a clean 1 litre separating funnel. One hundred ml. concentrated ice-cold ammonia are added and the mixture shaken. The lower ammoniacal layer is run off into a flask and the amyl alcohol layer washed with 100 ml. distilled water. The lower layer is run off into a separate flask and the amyl alcohol, which now contains little but haematin, discarded to residues.

The chief impurity present in these ammoniacal solutions of cruoratin appears to be protohaematin and its removal constitutes the chief barrier to further progress. There does not appear to be much bile-pigment present, or substance which will readily yield any of the recognized bile-pigments. Many attempts have been made to separate haematin and cruoratin by chromatography but as yet the degree of separation obtained has been of comparatively little help in view of the latter's instability.

It is less readily adsorbed than haematin on calcium and magnesium carbonates and the specimens used in the physical investigations were obtained by passing the ammoniacal solutions mentioned above down short columns of these solids. Insufficient other materials are available for the thorough chromatographic investigation which must precede other studies.

THE PROPERTIES OF CRUORATIN.

As prepared above cruoratin has certain characters which distinguish it from the haematins. It is much less stable, particularly towards oxidizing agents. Oxygen destroys it in solution, possibly due to peroxide formation from accompanying

substances. Potassium ferricyanide irreversibly oxidizes it to a tawny subtance with no well-marked spectroscopic bands; so does hydrogen peroxide even in low concentration. Acids have a similar destructive effect. In the absence of salts it is somewhat soluble in water and hence precipitation by acetic acid leads only to a progressively more impure specimen. In its ferric form it does not appear to combine with iminazole, cyanide or tertiary amines. Its solution in concentrated ammonia is greenish with a vague band at about 606 mμ while in sodium carbonate solution the colour is more tawny and the band absent, which is perhaps a hint of an ammoniacal compound. When dissolved in acid amyl alcohol it is clearly distinguished from haematin by the more crimson colour of the solution, the absorption band near the orange and the absence of marked absorption in the green and blue portions of the spectrum.

TABLE 2.

Maximum of visible bands of derivatives of cruoratin, measured with a Hartridge spectrometer.

Substance.	Solvent.		Remarks.
Cruoratin	NaOH	650	Vague.
Cruoratin	Ammonia	606	Weak, variable.
Cruoraem	Ammonia	610	Unstable.
Cruoraem CO	Ammonia	613	Increases after preparation.
Cruoraem CO + denatured ox globin	Ammonia	620·5	
Cruoratin	CH_3COOH / C_2H_5OH	610	Acid form; red solution.
Cruoratin	CH_3COOH / $(C_2H_5)_2O$	612	Neutral form; green solution.

Cruoratin is more readily reduced than haematin and in the reduced form can be dissolved in organic solvents. Reduced cruoratin ("cruoraem") combines with carbon monoxide but apparently not with nitrogenous substances such as pyridine or with cyanide. No evidence could be found for any specific compound of cruoratin or cruoraem with native horse globin. The position of the visible band of carbon monoxide cruoraem changes after preparation towards the infra-red, apparently due to some oxidative change.

As previously shown, the spectrum of fresh cruoralbin includes a medium-sized Soret band in the near ultra-violet. This has been confirmed. When cruoralbin is heated with dilute acid at 37° C. in order to detach the "labile iron" a curious and as yet inexplicable fact is observed. The intensity of the Soret band increases, despite the destruction of much of the cruoralbin. This increase is quite apparent from a direct comparison of the solutions, but when allowance is made for the liberation of some of the iron present the increase becomes striking.

TABLE 3.

The effects of heat and dilute acid on the ultra-violet spectrum of cruoralbin, photographed as the carbon monoxide compound.

Substance.	λ, mμ.	$\epsilon 10^{-2}$.	Remarks.
Cruoralbin 1	415	78	Native
„ 1	415	73	Denatured by alc. NH_3
„ 2	415	89	Fresh
„ 2	410	144	Heated
„ 3	415	76	Fresh
„ 3	410	129	Heated

The ε are based on the iron analyses.

TABLE 4.

The ultra-violet spectra of cruoratin derivatives.

Substance.	Solvent.	λ, mμ.	$\epsilon 10^{-3}$.
Cruoratin	Ammonia	402	107
Cruoratin	Acetic acid and ethanol	390	72
Cruoraem CO	Ammonia	408	141
Cruoraem CO and denatured globin	Ammonia	408	136

The ultra-violet spectra of cruoratin derivatives were measured. The band of carbon monoxide cruoraem is very intense and has a wave-length of about 7 mμ shorter than that of carbon monoxide haemoglobin. That of acid cruoratin is reminiscent of that of acid haematin, being lower, broader, and of a still shorter wave-length. It probably indicates a state of aggregation of the molecules.

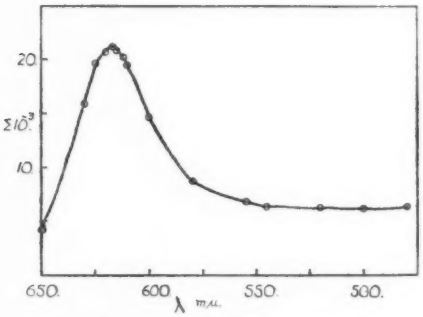

Fig. 1. Abscissae: wave-lengths in mμ.
Ordinates: $\epsilon 10^{-3}$.

ε is the equivalent absorption coefficient per gm. atom of iron.

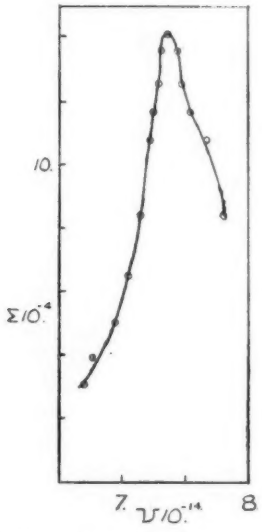

Fig. 2. Abscissae: $\nu 10^{-14}$ where ν is the frequency.
Ordinates: $\epsilon 10^{-4}$.

ε is the equivalent absorption coefficient per gm. atom of iron.

The visible absorption curve of carbon monoxide cruoraem was determined on a photo-electric spectrophotometer. Its band so nearly resembles that of carbon monoxide cruoralbin, previously published, that it would appear certain that most of the pigment in the latter substance is essentially cruoraem. This adds to the difficulty of the interpretation of the ultra-violet spectra.

After the extraction of the cruoratin the dark mass of protein residue was placed in a 300 ml. flask and 150 ml. of formic acid (90 p.c.) and some iron powder added. The flask was heated for an hour on a boiling water-bath to dissolve the protein and then refluxed for several hours. The contents were twice extracted with chloroform and centrifuged. The united chloroform extracts were

repeatedly extracted with normal hydrochloric acid to remove the porphyrin present. To the residual yellow solution in chloroform alcoholic zinc acetate and ammonia were added. A bright green fluorescence was observed indicating the presence of some substance related to urobilin. The vague absorption band at about 480–500 mμ became sharper and shifted to 504–508 mμ. A similar treatment of a cruoratin solution did not yield a similar result.

DISCUSSION.

The evidence presented suggests that cruoralbin consists, when pure, of a prosthetic group, cruoratin, joined to a protein. Neither cruoralbin nor cruoratin yield appreciable quantities of bile-pigments. Apparently a little bile pigment is formed during the preparation of cruoralbin, but it remains so attached to the protein that customary methods do not separate them. Cruoratin, though it appears to retain an intact porphyrin ring, is very unstable, being readily destroyed either by oxidizing agents or mineral acids. No evidence for the production of bile pigments during its destruction could be found. It probably represents a stage on a course of haemoglobin disintegration distinct from, and alternative to, the path that leads to urobilin.

SUMMARY.

The separation from cruoralbin of its prosthetic group, "cruoratin" is described. Its ultra-violet spectrum suggests that it possesses an intact porphin ring. Its only compound so far found is a carbon monoxide compound with its reduced form. Cruoratin is very unstable, being rapidly destroyed by oxidizing agents and mineral acids.

Acknowledgments. The author is indebted to Mr. A. T. Dick of the Council for Scientific and Industrial Research for the determination of a visible absorption curve.

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APPENDIX.

DETAILS OF SOLUTIONS FOR THE ABSORPTION CURVES.

Fig. 1. Stock solution contains cruoratin equivalent to 45·2 mg. of iron per litre. 0·75 ml. stock, 8 ml. N. NH_3, H_2O to 25·0 ml. saturated with coal gas. Then a little solid $Na_2S_2O_4$ added under coal gas.

Fig. 2. Stock solution contains cruoratin equivalent to 95·8 mg. of iron per litre. 1·0 ml. stock, 3·5 ml. N. NH_3 saturated with coal gas. Then under coal gas was added 0·5 ml. of a solution of 5 p.c. $Na_2S_2O_4$ in N. NH_3.

ANALYSIS OF SUCROSE FERMENTATION BY YEAST AT 0°C.

WITH SOME REMARKS ON "SELECTIVE FERMENTATION"

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Beta-d-fructopyranose is the only modification of d-fructose known in the crystalline state. On solution in water β-d-fructopyranose exhibits mutarotation which for the most part consists in a pyranose ⇌ furanose interconversion, a β-fructopyranose ⇌ α-fructopyranose change not being detectable by the methods available (Hudson, 1930; Isbell and Pigman, 1938). Both the rate of mutarotation of d-fructose and the position of the equilibrium are dependent on temperature. In experiments at 0° C. and at pH 4·5 under which conditions mutarotation proceeds most slowly, it was found that β-d-fructopyranose is unfermentable by yeast while α-d-glucose is utilized at the expected rate (Gottschalk, 1934a). Since in any quantitative consideration of the composition of a d-fructose solution at equilibrium and pH 4·5 open chain forms (enolform, ketoform) can be neglected (cf. Gottschalk, 1943b), d-fructofuranose, present mainly in the β-configuration (Ohle, 1927), remains as the only fructose form suitable for alcoholic fermentation by yeast. In order to prove the validity of this conclusion, further fermentation experiments at 0° C. and at pH 4·5 were carried out using, however, sucrose instead of β-d-fructopyranose as substrate. Conditions were so arranged that 50 p.c. of the disaccharide added was inverted by yeast saccharase to α-d-glucopyranose and β-d-fructofuranose in less than 1½ minutes. After fermentation periods of 20 and 40 minutes respectively at 0° C. the residual mixture of hexoses was analysed for total sugar and aldose in order to calculate the amounts of glucose and of fructose consumed. These experiments were supplemented by others at 0° C. in which the consumption by baker's yeast of sucrose was compared with that of α-d-glucose within a range of concentrations where the rate of fermentation is dependent on the concentration of the substrate.

EXPERIMENTAL.

The procedure of the experiments performed at 0° C. in a refrigerated laboratory and making use of a refrigerated centrifuge was similar to that described previously (Gottschalk, 1943a). Great care was taken that the temperature of the yeast suspension was adjusted exactly to 0° C. ± 0·05 before the substrate was added. Immediately after the addition of the sugar the cups, containing the yeast suspension and packed in snow within a big thermos flask, were stoppered, the thermos flask closed and continuously shaken (80 times p.m.) during the experiment. Baker's yeast (Effront), stored for 24 hours at 1–2° C. after having been compressed, was used. The yeast was suspended in 0·022 M KH_2PO_4 solution, unless otherwise stated. The pH of the suspension was in every case 4·5 at the beginning of the experiment. All experiments were carried out under anaerobic conditions which were ensured by equilibrating the yeast suspension and the gas space with carbon dioxide containing less than 0·1 p.c. oxygen. Each experiment was run with a control without sugar.

The sucrose added as substrate was pure; $[\alpha]_{5461}^{20°} = 78·3°$ (c = 10); a 0·3 p.c. solution did not reduce potassium ferricyanide when submitted to the Hagedorn-Jensen method. The α–d–glucose was the preparation used in the previous experiments.

In both sets of experiments with sucrose as substrate for fermentation no sucrose was detectable at the end of the experiments, as was seen from a comparison of the reducing power of the supernatant before and after acid hydrolysis; this hydrolysis was performed according to Jackson and Gillis (1920). In the experiments of the first series the supernatant (obtained as described previously) was analysed for total reducing power (glucose plus fructose) and for

aldose, the difference indicating the amount of fructose present. The total sugar left at the end of the experiments was determined in the clear supernatant in triplicate by the Hagedorn-Jensen method using a microburette which could easily be read to 0·005 ml. Advantage was taken of the recommendations in the paper by Kramer and Steiner (1931). To allow for the difference in reducing powers of glucose and of invert sugar a correction was applied. The exact value of this correction was obtained by determining the reducing power of different concentrations of invert sugar derived from sucrose by acid hydrolysis (cf. above). After inversion for analysis the pH of the invert sugar solution was adjusted to about 4·0 (pH value of the yeast suspension at the end of the experiment). The thiosulphate equivalent of invert sugar in the concentration concerned is 0·95 referred to glucose as unity.

The aldose determination was performed by the iodimetric method in the modification recommended by Macleod and Robison (1929). We have satisfied ourselves that by this method 1 mg. glucose is completely oxidized in 30 minutes at 22° C. (waterbath), while the values obtained for fructose are of practically the same magnitude as the titration error of this method. The iodimetric aldose determination has to be preceded by the removal from the supernatant of all compounds other than glucose which may react with iodine under these conditions. The application of dialysed iron (liquor ferri dialysatum) followed by the removal by distillation of volatile substances gave very satisfactory results. By adding 2·0 ml. dialysed iron and 0·55 ml. 1 N Na_2CO_3 to 17·5 ml. supernatant a clear filtrate (pH = 7·0) free from protein and iron was obtained. This filtrate was concentrated to one-third of its volume in the vacuum of a mercury pump with the distilling flask left unheated and the condenser cooled in ice. On this liquid the aldose determination was carried out in triplicate. When the supernatant was treated in this way, 11·70–11·75 ml. 0·005 N sodium thiosulphate were required for titration in the control experiment (yeast suspension without sugar) as compared with 12·0 ml. when the iodine solution was directly titrated. To test the accuracy of the method known amounts of glucose were added to the centrifuged supernatant from a suspension of yeast in phosphate solution and the analysis carried out in the manner described. 95 p.c. of the glucose added was recovered. The experimental results obtained were corrected accordingly.

The sucroclastic activity of the yeast cells under the conditions of the experiments of the second series was estimated by determining the amount of invert sugar present in the yeast suspension after a short period of action. As a rule 1·0 gm. yeast was suspended in the appropriate volume of KH_2PO_4 solution (cf. Table 2) and cooled down to 0° C.; after the admixture of 15–20 mg. sucrose this suspension was shaken for 5–7 minutes at 0° C., when the inversion was stopped by the addition of 0·15 ml. 60 p.c. KOH, thus bringing the pH of the sample to 8–9. A control with α–glucose as substrate served to allow for any fermentation during the course of inversion. By means of a few experiments, carried out in this manner with each yeast sample, the period of half-inversion, i.e. the number of minutes necessary for the inversion of half the amount of sucrose added, was determined. Since the rate of inversion is directly proportional to the concentration of yeast saccharase over a wide range of enzyme concentrations (Nelson and Vosburgh, 1917), the value of the period of half-inversion in the main experiment with 5 gm. yeast (second series) could be calculated from the results of these preliminary tests. The average period of half-inversion thus determined was found to be 1·2 minutes, a value which was repeatedly checked by experiments at 0° C. with 5·0 gm. yeast and 15·0 mg. sucrose interrupted after 65–75 seconds. In the experiments of the second series, to provide comparable conditions, the experiment using sucrose was started 2½ minutes earlier than that with glucose. The longer fermentation time in the sucrose experiment tended to compensate for the effect on the fermentation rate of the lower initial concentration of total hexoses which ensued from the fact that only 75 p.c. of the sucrose added was inverted when the corresponding experiment with glucose started. The times in Table 2 are measured from the beginning of the glucose experiments.

The dry weight of the yeast was estimated as before (Gottschalk, 1942).

The experimental results are expressed in milligrams of hexose per total volume assuming that total water (solution plus yeast water) is solvent and that the sugar is uniformly distributed in the solvent.

All pH measurements were made with the glass electrode.

RESULTS.

The experimental results are recorded in Table 1 and Table 2.

It may be seen from the figures in Table 1 that approximately equal amounts of d–fructose and d–glucose are left when the fermentation of sucrose at 0° C. is interrupted after 20 or 40 minutes. If, however, the experiment is carried out at 25° C. with 0·2 gm. baker's yeast instead of using 5·0 gm. yeast at 0° C. and the fermentation stopped after 25 minutes, the residual mixture of hexoses, differentiated in the same manner as above, consists of about 65 p.c. d–fructose and 35 p.c. d–glucose (Table 1, Nos. 15 and 16). Similar results are obtained when a mixture of equal parts of d–glucose and d–fructose is partly fermented at 25° C. (for literature cf. Sobotka and Reiner, 1930).

TABLE 1.

Analysis of sucrose fermentation by yeast at 0° C.

Conditions: 6·0 gm. baker's yeast suspended in 23·0 ml. 0·022 M KH_2PO_4 solution; saturated with CO_2 at 0° C. Substrate: sucrose. Temperature: 0° C. ± 0·05. Time: 20 min. (Nos. 1–7) and 40 min. (Nos. 8–14) respectively. Gas phase: CO_2. Experiments 15 and 16 were carried out with 0·2 gm. baker's yeast at 25° C. in nitrogen; fermentation interrupted after 25 minutes.

No.	Sucrose added (calculated as invert sugar). mg.	Glucose plus fructose recovered. mg.	Glucose recovered. mg.	Fructose recovered (by difference). mg.	Percentage of glucose consumed.*	Percentage of fructose consumed.*
1	20·4	12·7	6·4	6·3	37·3	38·2
2	20·0	14·6	7·3	7·3	27·0	27·0
3	21·4	15·5	7·9	7·6	26·2	29·0
4	20·6	15·5	7·8	7·7	24·3	25·2
5	20·6	14·0	6·9	7·1	33·0	31·1
6	20·8	15·3	7·6	7·7	26·9	26·0
7	20·0	14·1	7·0	7·1	30·0	29·0
8	19·8	10·0	5·0	5·0	49·5	49·5
9	20·2	10·1	5·2	4·9	48·5	51·5
10	20·1	9·8	5·0	4·8	50·2	52·2
11	20·2	10·0	4·9	5·1	51·5	49·5
12	20·0	10·8	5·4	5·4	46·0	46·0
13	20·1	8·8	4·5	4·3	55·2	57·2
14	20·0	10·0	4·8	5·2	52·0	48·0
15	32·1	18·0	6·4	11·6	60·1	27·7
16	40·5	22·9	8·6	14·3	57·5	29·4

* Calculated according to the formula $\frac{\text{Hexose consumed} \times 100}{0{\cdot}5 \times \text{invert sugar added}}$.

TABLE 2.

Comparative consumption by baker's yeast of α–d–glucose and of sucrose in low concentrations at 0° C.

Conditions: 5·0 gm. baker's yeast suspended in 31·0 ml. 0·022 M KH_2PO_4 solution (Nos. 1–6) and 5·0 gm. baker's yeast suspended in 23·5 ml. 0·050 M KH_2PO_4 solution (Nos. 7–14) respectively; saturated with CO_2 at 0° C. Substrates: α–d–glucose and sucrose. Temperature: 0° C. ± 0·05. Time: 20 min. (Nos. 1–6) and 40 min. (Nos. 7–14) respectively. Gas phase: CO_2.

No.	Glucose added. mg.	Glucose recovered. mg.	Sucrose added (calculated as invert sugar). mg.	Glucose plus fructose recovered. mg.	Percentage of glucose consumed.	Percentage of of glucose plus fructose consumed.*
1	15·5	11·0	15·7	10·3	29·0	34·4
2	15·1	11·4	15·0	10·7	24·5	28·7
3	15·1	11·0	14·9	9·7	27·2	34·9
4	16·1	11·1	16·4	10·4	31·1	36·6
5	15·9	12·4	16·1	11·8	22·0	26·7
6	15·3	11·5	15·8	11·0	24·8	30·4
7	20·0	9·2	20·1	8·6	54·0	57·2
8	20·2	10·5	20·2	10·2	48·0	49·5
9	19·9	10·6	20·0	9·7	46·7	51·5
10	20·2	11·4	20·1	10·4	43·6	48·3
11	19·9	8·6	19·9	8·8	56·8	55·8
12	20·2	8·1	20·2	7·4	59·9	63·4
13	20·0	10·3	20·0	10·4	48·5	48·0
14	20·0	9·2	20·1	8·3	54·0	58·7

* Calculated according to the formula $\frac{\text{Hexoses consumed} \times 100}{\text{invert sugar added}}$

The experimental results shown in Table 2 are in close agreement with those in Table 1. Comparing the consumption by baker's yeast at 0° C. of α–d–glucose in 0·0024 or 0·0040 M concentration with that of α–d–glucose plus β–d–fructofuranose each in 0·0012 or 0·0020 M concentration, as derived from sucrose by the saccharase of the yeast, it is found that either about equal amounts of d–glucose and of the glucose-fructose mixture are fermented or some per cent. more of the latter mixture. From independent fermentation tests carried out at 0° C. with α–d–glucose in 0·0020–0·0040 M concentrations we have seen that within this range the rate of fermentation is dependent on, but not directly proportional to, the substrate concentration, as was to be expected from the results of previous experiments at 25° C. (Gottschalk, 1944). Furthermore it may be mentioned that with substrate concentrations high enough to saturate the enzyme molecules of the limiting reaction, the relative rates of CO_2 production by yeast from d-fructofuranose and from d–glucose at 0° C. are 105 and 100—the same as at 25° C. This was ascertained by experiments in which the volumes of CO_2 produced at 0° C. from 0·1 M α–d–glucose and from 0·1 M raffinose, split by baker's yeast into fructofuranose and the unfermentable melibiose, were measured. The possibility of a direct fermentation of sucrose, i.e. without a preceding inversion, as favoured by Willstätter and Lowry (1925), can be ruled out under the experimental conditions adopted in this paper, since already after 70–80 seconds half of the sucrose added is recoverable as invert sugar (cf. also Cohn, 1927; Toropova, 1939).

DISCUSSION.

While it was previously shown that d-fructopyranose added to yeast at 0° C. is unfermentable, the sugar balances in Table 1 are evidence that d-fructofuranose, set free from sucrose by yeast saccharase, is readily fermented under the same conditions. If fructofuranose and glucose compete for hexokinase, as they do in the fermentation of sucrose, the relative rates of fructose and of glucose disappearance from the medium depend on the relative concentrations of fructofuranose and glucose inside the yeast cell and their relative affinities for the common enzyme. Concerning the actual concentration of fructofuranose available for fermentation it has to be borne in mind that part of the fructofuranose liberated from sucrose is converted reversibly to fructopyranose (cf. Fig. 1). Mutarotation of d-fructose at 0° C. is by no means negligible. From the data available (Nelson and Beegle, 1919; Gottschalk, 1943b) it can be calculated that at 0° C. from 10 mg. d-fructofuranose, dissolved in a buffer solution of pH 4·5, after 20 minutes 3·3 mg., after 40 minutes 5·0 mg. would be present in the pyranose form, the ratio $k_1 : k_2$ of the rate constants of the forward and of the back reaction being about 7. The fact that in the fermentation of sucrose the consumption of fructofuranose, despite the depressing effect of mutarotation on its actual concentration, equals that of glucose is proof that fructofuranose has a higher affinity for hexokinase than has glucose.

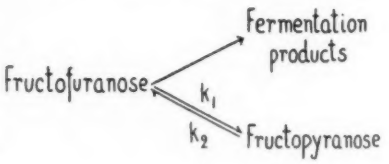

Fig. 1.

The difference in the relative amounts of glucose and of fructose left after partial fermentation of sucrose by baker's yeast at 0° C. and at 25° C. is due to the circumstance that under the conditions adopted at 0° C. the interconversion to fructopyranose of part of the fructofuranose, liberated from sucrose, is far from complete after 20 minutes, while at 25° C. equilibrium is already nearly established after 12 minutes. Therefore, the ratio of the competing fermentable hexoses glucose/fructofuranose is on an average much greater in the experiments at 25° C. than in those at 0° C. The "selective fermentation" at room temperature of glucose from invert sugar by yeast, referred to in the literature (Willstätter and Sobotka, 1922; Hopkins, 1928), is the result of the unequal distribution of hexokinase between its substrates d-glucose and d-fructofuranose; the aldehyde sugar seizes more enzyme molecules, since its higher concentration within the yeast cell outweighs the greater affinity of fructofuranose for hexokinase.

Assuming that in the fermentation by yeast of a mixture of d-glucose and d-fructose the rate of fermentation of each individual sugar is proportional to its concentration at any time, the results of experiments on "selective fermentation" are usually expressed by the formula $\frac{k_g}{k_f} = \frac{\ln a_o - \ln a}{\ln b_o - \ln b}$, where a_o and b_o are the initial concentrations of glucose and of fructose, a and b the concentrations of the respective sugars at any given time and where $\frac{k_g}{k_f}$ is the ratio of the separate rate constants of glucose and of fructose consumption from a mixture of both by yeast. It may be pointed out that the application of this formula is not permissible, because in the fermentation of an equilibrated solution of invert sugar the change in concentration of the fermentable fructofuranose, competing with glucose for hexokinase, results from the formation of fermentation products *and* from the reconversion of part of the pyranose to the furanose form (cf. Fig. 1). If the fermentation rates, both of d-glucose and of d-fructofuranose, were directly proportional to the substrate concentrations, the first order rate constant of glucose fermentation could be calculated according to the equation of a first order reaction and that of d-fructofuranose fermentation would be obtainable by a linear second order differential equation with constant coefficents covering the case, represented in Fig. 1, of two simultaneous reactions of the first order, one of which is reversible. We have found, however, that even with a glucose concentration as low as 0·002 M alcoholic fermentation (at 0° C.) does not conform to the equation of a first order reaction.

The experimental results set out in Table 2 support those in Table 1. Though in the sucrose experiment owing to mutarotation the total concentration of fermentable hexoses is at any instant lower than in the glucose experiment, the sugar consumption using sucrose as substrate is at least as high as that using glucose. Since with the sugar concentrations employed the rate of fermentation is dependent on the substrate concentration, this result affords further evidence that d-fructofuranose has a greater affinity for hexokinase than has d-glucose. For the reasons given above the ratio of the affinities cannot be calculated from the results of this paper; but the figures in Tables 1 and 2 conform to the previous finding that the ratio of the dissociation constant of the hexokinase-glucose complex to that of the hexokinase-fructofuranose complex is approximately 2 (Gottschalk, 1944).

SUMMARY.

0·002 M sucrose in phosphate solution (pH 4·5) was fermented by baker's yeast at 0° C. with conditions so arranged that 50 p.c. of the disaccharide added was already inverted within 70–80 seconds. It was found that after fermentation periods of 20 or 40 minutes approximately equal amounts of d-glucose and d-fructose are left in contrast to the preponderance of d-fructose in the residual aldehyde-ketose mixture when sucrose is fermented at 25° C. The equality, in sucrose fermentation at 0° C., of the consumption of α-d-glucose and of β-d-fructofuranose in spite of the interconversion of part of the fructofuranose to the unfermentable pyranose form (mutarotation) indicates that fructofuranose has a higher affinity for hexokinase than has glucose. Further experiments at 0° C. in which the consumption by baker's yeast of sucrose was compared with that of α-d-glucose within a range of concentrations where the rate of fermentation is dependent on substrate concentration confirmed the above result. The "selective fermentation" of glucose from invert sugar at room temperature is interpreted on the basis of the relative concentrations of d-glucose and d-fructofuranose inside the yeast cell and of their relative affinities for the common enzyme hexokinase.

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THE ROLE OF BORON IN PLANT METABOLISM

3. THE INFLUENCE OF BORON ON CERTAIN ENZYME SYSTEMS.

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Plant enzyme systems influenced by boron might be detected in the following ways:

1. Symptoms caused in entire plants by deficiency or excess of boron might be used in deducing the probable enzyme system involved. The behaviour of this enzyme, its substrate or its products could then be studied in the presence of a series of boron concentrations.
2. If a tissue is found whose respiration is inhibited by a moderate excess of boron in the medium, then by trial with known cell constituents, compounds might be found whose addition to the medium would annul the inhibition. Knowledge of the properties of these compounds would then enable deduction of the enzyme system which was inhibited.
3. Of the enzyme systems known to occur in the cell, those whose sensitivity to boron is theoretically possible could be studied in the presence and absence of boron.
4. Comparison could be made of the biochemistry of two organisms, one of which requires boron for its growth and the other does not. Any enzyme systems occurring exclusively in the former organism might be suspected of requiring boron as a component.

Attempts to employ these methods are described in the present paper and some have been described already in the other papers in this series. The first method suffers from the great disadvantage that the symptom observed is not the direct effect of the excess or deficiency of boron but is separated from it by a chain of unknown reactions. To minimize this difficulty Alexander (1942) attempted to observe short-time responses to boron deficiency. He found that one such effect in squash plants was an increase in catalase activity. Unfortunately this is difficult to interpret, epecially because it was not indicated whether the actual amount of catalase increased. It has been mentioned previously (Smith, 1945; Winfield, 1945) that the likelihood of a connection between boron, calcium and pectic compounds was indicated by boron deficiency symptoms in higher plants. The results of Herzinger (1940), if confirmed, would be of great significance; he claimed that boric acid in certain concentrations promoted the reducing action of an enzyme which he called reductase, and repressed the activity of tyrosinase, thus preventing melanin formation. Herzinger was dealing with potato cubes and had no good reason for ascribing the phenomena to specific enzymes. The fourth method assumes increasing importance with the mounting knowledge of plant biochemistry, but is obviously limited until most or all of the enzyme systems of the two organisms compared are known. A great simplification would be possible if two bacterial species could be compared and it is indicated later that there may be bacteria which require boron for their growth and others that do not.

As far as can be ascertained the only reported enzymatic action which is related to boron metabolism, and which has not been mentioned elsewhere in this series of papers, is one in which sulphate esters are hydrolyzed to sugars by glucosulphatase, an enzyme occurring in molluscs (Soda, 1936).

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EXPERIMENTAL RESULTS.

The Supposed Influence of Boric Acid upon the Action of Potato Aldehyde Oxidase and Tyrosinase.

The only enzyme from potato which could correspond to Herzinger's (1940) hypothetical "reductase" seemed to be aldehyde oxidase. The latter was therefore isolated in pure form from potato tubers and permitted to reduce nitrate to nitrite by means of acetaldehyde in buffered (phosphate, pH 7·2) solutions containing various amounts of boron added as boric acid. After incubation for two hours at 37° C. nitrite was determined by means of sulphanilic acid and α-naphthylamine (Snell and Snell, 1936). The amount of nitrite formed was identical in solutions containing 0, 0·1, 1·0, 10, 100 and 500 p.p.m. of boron.

A number of impure tyrosinase preparations, obtained by partial isolation of the enzyme by repeated alcohol precipitation from potato tubers of several varieties as well as from squash leaves, sugar beets and sweet-potato tubers, were tested for tyrosinase, catecholase and cresolase activity in the presence of a range of boron concentrations at a number of pH levels between 5·0 and 8·0 in phosphate buffer In a few instances a response to boron was observed, for example an increasing repression of tyrosinase activity by increasing boron concentrations from 0·01 to 1,000 p.p.m., and in another case a maximum catecholase activity in the presence of 0·01 p.p.m of boron with a second but lower maximum at 1,000 p.p.m. The rate of oxidation of m-cresol was remarkably constant at all boron concentrations up to 1,000 p.p.m. Where responses were obtained in the above experiments they were mostly of small magnitude and not reproducible. It is therefore necessary to conclude that boron probably has no influence upon aldehyde oxidase, tyrosinase, catecholase and cresolase activity *in vitro*, at least in the presence of phosphate and in the pH range 6 to 8.

An explanation of Herzinger's results became evident when the latter's experimental technique was followed as closely as possible. When Herzinger's procedure for reduction of methylene blue by potato cubes was carried out in open test-tubes, and in absence of buffer, the effects corresponded approximately to those obtained by the above author, with a maximum reduction of methylene blue in the presence of 1 p.p.m. of boron. However the results from one experiment to another were erratic. No maximum at 1 p.p.m. of boron could be detected when the solutions were buffered at pH 6·0 by phosphate. In no case did any reduction occur within twenty-four hours, and generally it was not until thirty-six hours had elapsed that an appreciable amount of leuco-methylene blue was formed. When the experiments were conducted in Thunberg tubes in the absence of air no reduction occurred. Where reduction of methylene blue did occur in the above experiments (and possibly in Herzinger's) it was actually performed by bacteria and not by the potato tissue. The organisms could be seen as a white turbidity, and on microscopic examination appeared to be aerobic spore-formers. Presumably these bacteria are aerobes whose growth is stimulated by boron concentrations of the order of 1 p.p.m. and strongly inhibited by 1,000 p.p.m. Herzinger's procedure again was followed closely in the case of melanin formation by potato cubes in a series of boron concentrations. No consistent effects could be obtained although in some experiments there was an approximate concordance with Herzinger's results. Any responses were due almost certainly to pH effects (tyrosinase is sensitive to pH changes in the range 6 to 8) and accidental bacterial growth. In the presence of phosphate buffer there was usually no variation in the rate of production of red and black pigment by potato cubes in the various boron treatments.

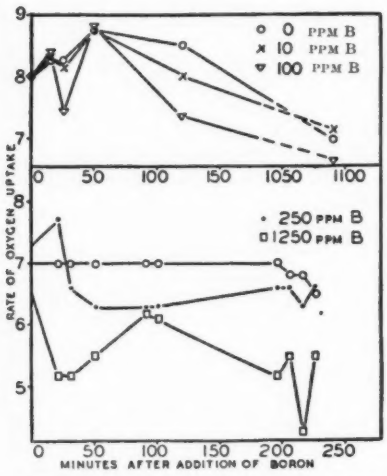

Fig. 1. Respiration of boron deficient tomato roots in solutions of different boron concentrations.

The Influence of Boron on Respiration.

The first tissue whose respiration was examined for response to boron was the root of boron-deficient tomato. Tomato seedlings reared in a boron-deficient culture solution (Smith, 1943a) grew to a height of almost two inches, when death occurred of the apical meristems. Two days after appearance of boron-deficiency symptoms in the roots the latter were excised, cut into 1 cm. lengths and mixed thoroughly before being placed in the vessels of a Fenn apparatus. Each vessel contained 3·0 ml. of the boron-deficient culture solution, 0·3 ml. of 10 p.c. potassium hydroxide solution in one side-arm, and in the second side-arm 0·3 ml. of either water or a boron solution (boric acid buffered to the same pH as the culture solution) which was added to the

culture-solution at 4 p.m. by tipping. Readings of the distances moved by the oil-drop of each manometer were taken at ten minute intervals. Actual rates of oxygen consumption were not calculated. The rates shown in Fig. 1 are expressed in terms of a common initial rate of 8·0 arbitrary units for the average rate of each pair of the three treatments. Zero time in the lower half of Fig. 1 corresponds to the addition of more boron 1,160 minutes after the beginning of the experiment. It will be seen that a boron concentration of 10 p.p.m. did not influence the respiration rate, while 100 p.p.m. resulted in only a weak inhibition. The response to 250 p.p.m. of boron was likewise feeble and even 1,250 p.p.m. caused an inhibition of only 15–20 p.c.[2] In a period of twenty-three-hours there was in no treatment any appreciable stimulation of the respiration of boron-deficient tomato roots by addition of boron. This surprising result was in strong contrast with the results of experiments with intact boron-deficient tomato plants in which new root growth was visible twenty-four hours after addition of boron to the culture solutions. Possibly the concentration of boron within the cells of the roots in the respiration experiments never reached a high level, due either to exudation of boron almost as fast as it was absorbed, or to an extremely slow absorption rate attributable to weak respiration (indicated by earlier exudation experiments, and by other respiration studies in this Division) and the absence of transpiration. It has been shown previously (Smith, 1943b) that tomato roots do not greatly increase their boron concentration when placed in solutions containing a high concentration of this element.

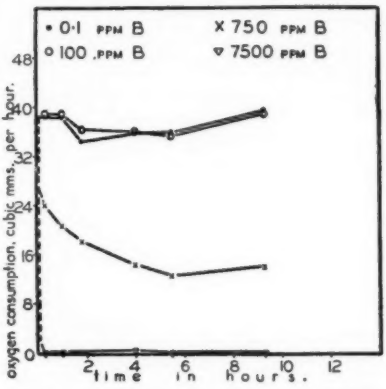

Fig. 2. Inhibition by boron of the respiration of normal barley roots.

Barley roots were chosen as a more suitable material for further study, having in mind that barley, with a much lower boron requirement than tomato, should be more sensitive to an excess. The technique for preparing the barley roots and following their respiration was precisely that of Machlis (1944). Each vessel of the Fenn apparatus contained six pieces of root including no root-tips. Boron was added at 2 p.m. and readings taken at ten minute intervals. The average rates of oxygen uptake at six intervals during the first ten hours are presented in Fig. 2, where it is shown that 750 p.p.m. of boron induced a 37 p.c. inhibition of respiration within twenty minutes. After four hours the inhibition reached 60 p.c. This is a far greater response than that of the boron-deficient tomato roots to 1,250 p.p.m. of boron. 7,500 p.p.m. of boron reduced the respiration to zero in less than ten minutes. The concentration of 100 p.p.m. had no appreciable effect, although a slight stimulation of respiration in the first two or three hours is indicated in Fig. 2.

Even though the barley roots proved much more subject to boron toxicity than the previously used roots, they are not entirely satisfactory for the type of study mentioned in method 2 of the introduction, because a concentration of about 500 p.p.m. of boron would be required to induce respiratory inhibition of a convenient magnitude. 0·1 molar then would be the minimum concentration of sugar, organic acid or other compound which could be added to annul the inhibition. In spite of the high concentrations involved it may be possible to gain useful information by this method. However more promise lies in the utilization of certain bacteria, which are susceptible to much lower boron concentrations than are barley roots, once the species and suitable cultural methods for the bacteria are established. The experiments of the previous section indicated that some aerobes are sensitive to boron concentrations of the order of 1 p.p.m., and, although there has been no indubitable proof that boron is an essential element for certain bacteria, Herzinger (1940) has shown that it is probably necessary for a number of microorganisms such as *Azotobacter chroococcum* and *Clostridium pasteurianum*.

The Influence of Boron on Phosphorolysis.

There are theoretical reasons (Winfield, 1945) for believing that boric acid could enter into condensation reactions of sugar molecules in the same fashion as does phosphate ion. It was

[2] The extraordinary tolerance by tomato plants of supposedly toxic boron concentrations was illustrated also in some experiments on the influence of boron toxicity on fruit production. Twelve tomato plants, grown in culture-solution containing 5 p.p.m. of boron in two 45 litre tanks for four months in the greenhouse, suffered marginal scorching of the older leaves, and matured earlier than similar plants which received 0·5 p.p.m. of boron. Nevertheless the yield of ripe fruit by the plants suffering from boron-toxicity was little inferior: 20·4 kilograms as compared with 23·6 in the case of normal plants.

therefore of interest to determine whether boric acid could compete with phosphate for sugar molecules in a known polysaccharide synthesis. The reversible synthesis of starch from glucose–1–phosphate was a convenient system to study because it could be carried out *in vitro*. Starch degradation was followed by analysis for the glucose–1–phosphate formed, and starch synthesis from Cori ester by determining the phosphate liberated, at 22° C. and employing the technique of Hassid and McReady (unpublished). Boron concentrations were 0·1 molar, obtained by addition of boric acid solutions which had been adjusted previously to the desired pH with potassium hydroxide. For the degradation of starch the solutions were made up to be 0·1 molar in phosphate buffer at pH 6·8. Determination of Cori ester was made at intervals for eighty minutes. The average acceleration of degradation in the presence of the borate was found to be 17 p.c. For starch synthesis the reaction was allowed to proceed in a citrate buffer of pH 6·0. Borate induced at 21 p.c. inhibition of the synthesis on the basis of phosphate produced in three minutes.

When it is considered that the molecular concentrations of phosphate and borate were equal in the case of the starch degradation, it seems unlikely that boron influences the starch $\rightleftharpoons$ glucose–1–phosphate equilibrium in the normal plant, where phosphate concentrations would be much greater than borate. This is especially true since chloroplasts have a relatively low boron concentration (Smith, 1944).

The Comparative Biochemistry of Organisms Differing in Their Boron Requirement.

Little is known about the relative boron requirements of different organisms apart from the higher plants. In the present studies it was found that deficiency symptoms could not be induced in *Aspergillus niger* and *Penicillium glaucum* grown in synthetic media low in boron. Previous experiments by Steinberg (unpublished) also had failed to demonstrate the necessity of boron for the growth of *Aspergillus niger*. The assumption will be made therefore that boron is not required for the growth of either of the above fungi. It is further assumed that they do not require calcium (Steinberg, unpublished; Raulin, 1863) although an exceedingly small amount may be needed for perhaps one of the several functions which calcium performs in higher plants. On this basis it should be possible to learn something of the function of boron, and also of calcium, in higher plants by a study of the comparative biochemistry of fungi such as *Aspergillus niger*. The problem resolves itself into the determination of which enzyme systems, that could be dependent upon boron, occur universally in higher plants and not in *Aspergillus niger* or similar organisms. The enzyme systems responsible for synthesis of pectic compounds are among the very few that fall in this category, as far as could be ascertained from a survey of the very limited literature dealing with fungal metabolism.

Mycelium of *Aspergillus niger* was extracted twice with boiling alcohol, and the residue ground thoroughly in a Waring Blendor yielding a mixture which was boiled and centrifuged prior to analysis of the filtrate. The mucic acid test for galacturonic acid was negative. Tollen's naphthoresorcinol test (Browne and Zerban, 1941) indicated the presence of a small amount of uronic acid, but the colour obtained was modified by the presence of a sugar, probably a pentose. A litre of the medium (the Czapek-Dox medium, Winfield, 1945) in which the fungus had grown was evaporated on the steam-bath to a small volume. With this solution Ehrlich's test (Browne and Zerban, 1941) for galacturonic acid was positive, and also the cobaltous chloride reaction for d–fructose. No galacturonic acid or pectic material could be detected in mycelium grown in a medium (Winfield, 1945) containing lactose as the sugar.

100 gm. fresh weight of *Penicillium glaucum* mycelium (grown in the Czapek-Dox medium) previously rinsed with water was ground in a Blendor with 500 ml. of water. Filtration through a Whatman No. 4 paper gave an extract (a) which had a grey turbidity. The residue from the above filtrations was blended with 400 ml. of water and boiled gently for an hour. While still hot the mixture was filtered through a No. 4 paper yielding a very turbid filtrate, from which precipitated during cooling a large amount of grey flocculent material (d) which was somewhat gelatinous. After cooling the latter mixture in the refrigerator it was filtered through a No. 42 paper to give a yellowish-grey solution (b) with a faint opalescence, and a residue of the grey material (d) whose constitution is unknown and deserves study. An extract (c) was obtained by boiling the residue from the second filtration with 60 ml. of saturated ammonium oxalate solution, and 300 ml. of water, followed by filtration. 10 gm. of sucrose were added to each of the extracts (a), (b) and (c) and they were evaporated on the steam-bath to a volume of 25 ml. Pectic acid determinations were then carried out according to the method of Wichman and Chernoff (Browne and Zerban, 1941). The percentages of pectic acid in terms of fresh weight of mycelium were 0 for extracts (b) and (c) and 0·003 for (a). The material responsible for the latter figure did not resemble pectic acid in physical properties so that it seemed safe to conclude that the mycelium contained no pectic compounds whatsoever. Another batch of mycelium was grown in the lactose medium, containing 1,000 p.p.m. of boron. After extracting the mycelium with boiling alcohol it was ground with water, then boiled and centrifuged. The extract obtained in this way was concentrated on the steam-bath, cooled and poured into acetone, yielding a brown curdy precipitate. The precipitation was closely comparable with the precipitation of pectin by acetone. A solution of the brown curdy precipitate gave exactly the same result in the naphthoresorcinol test for uronic acid as did a dilute pectin solution.

It appears then that *Penicillium glaucum* can produce a substance with some of the properties of pectin, and which contains uronic acid, when it is grown in the lactose medium containing excess boron.

Later it is hoped to determine conclusively whether the above two fungi are capable of synthesizing pectic substances, or polygalacturonides of any description, and whether their synthesis can be induced by boron, calcium or a suitable sugar in the medium.

DISCUSSION.

It seems likely that both *Aspergillus niger* and *Penicillium glaucum* are incapable of synthesizing true pectic compounds. Under certain conditions, for example when boron and galactose are provided, *Penicillium glaucum* may produce a type of polyuronide. *Aspergillus niger* is able to synthesize galacturonic acid in a medium containing glucose as the only sugar, but it is improbable that the galacturonic acid can be polymerized.

It should be noted that *Aspergillus niger* has considerable pectinase activity, so that no polygalacturonide can be expected in this fungus unless there is some mechanism for its protection, or an exceptionally high rate of synthesis. Leaves of higher plants generally contain pectase but no protopectinase or pectinase (Gortner, 1938). Pectase does occur in some fungi but possibly only in those which synthesize pectic compounds as part of their wall structure, e.g. *Pythium* (Thomas, 1942). This enzyme which demethoxylates pectin to pectic acid, and which occurs in the regions of vigorous growth of a large number of plants (Bertrand and Mallevre, 1895), has been known for many years without its function being understood. When it is remembered that a number of well-known esterases are reversible, and can be made to catalyze both the condensation and hydrolysis reactions of the same equilibrium, it seems possible that the role of pectase, in the meristems of plants which have pectic intercellular substance, is in methylation of carboxyl groups of the polygalacturonide constituent of the precursor of protopectin[3]. It should be interesting to study the influence of boron, calcium and pectase on the methylation of a polygalacturonide by a donor (e.g. choline) of the transferable methyl group.

The indication from the foregoing experiments that boron and pectic metabolism are related, though based on scanty evidence, is supported by a considerable body of literature which already has been reviewed (Smith, 1943b). There is also good reason to believe that boron and calcium metabolism are interdependent (Smith, 1945, and others cited there). It is desired to emphasize that calcium and pectic metabolism are closely related[4], and there is no way to determine whether it is calcium metabolism or pectic metabolism that is the more directly connected to boron metabolism.

The extensive survey of possible functions of boron presented in these three papers, though not conclusive, suggests that the most promising lines of investigation are *via* the relationship between boron and pyridoxin, and boron and pectic compounds.

SUMMARY.

A number of techniques have been tried for the detection of enzyme systems influenced by boron. Herzinger's (1940) claims that certain enzymes in potato tubers are sensitive to boron concentrations of the order of 1 p.p.m. could not be confirmed. Boron was found to have no influence on potato aldehyde-oxidase and tyrosinase *in vitro*. Herzinger's results may be attributed to contamination of the potato cubes by aerobic bacteria which require boron for their growth.

[3] It is assumed that the order of formation of pectic compounds in plants is protopectin → pectin → pectinate → pectate.

[4] The amount of calcium in solution in the cell can be controlled by the pectic compounds since pectinic and pectic ions precipitate calcium.

Boron even at high concentrations had practically no effect on the respiration rate of boron-deficient tomato roots. At no concentration was there a stimulation of respiration. High concentrations inhibited the respiration but even in the presence of 1,000 p.p.m. of boron the inhibition was weak. Normal barley roots proved to be more susceptible to additions of boron, but even with this tissue a concentration of 750 p.p.m. of boron was required to reduce by half the rate of respiration.

Synthesis of starch from glucose-1-phosphate was inhibited, and the reverse process of starch degradation accelerated, by boron, but the magnitude of the effect is considered insufficient to be important in normal plant metabolism.

A beginning has been made in the study of the comparative biochemistry of organisms differing in their boron requirement. *Aspergillus niger* and *Penicillium glaucum*, organisms which it is assumed do not require boron and calcium for their growth, appear to be incapable of synthesizing true pectic compounds. Under certain conditions the former species may synthesize galacturonic acid and the latter a polyuronide. These observations are in accord with the theory that boron, calcium and pectic metabolism in higher plants are related.

Acknowledgments. The studies described in the series of three papers were undertaken under the direction of Professor D. R. Hoagland, whom it is desired to thank together with all of the members of this Division, particularly Mr. C. C. Delwiche and the late Professor C. B. Lipman. The helpful criticism by Professor J. S. Turner of the Botany School, University of Melbourne, is gratefully acknowledged, likewise the generous support of the Hackett Fund of the University of Western Australia; the International House, Berkeley; and the Carnegie Corporation of New York.

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NOTE.

In the previous paper (Winfield, 1945) several examples were given of the types of chemical reaction which might be influenced by boron. Some recent experiments provide a further example which is particularly interesting because it involves the condensation of two or more dihydroxy short-chain molecules.

When mixtures of boric acid, 2, 3–butanediol (prepared by fermentation of wheat employing *Aerobacillus polymyxa*) and varying amounts of water were distilled at temperatures from 20° C. upwards, depending upon the pressure, the products contained a number of boric esters, the relative amounts of each apparently being governed by the temperature at which the distillation was carried out and the initial water concentration. The esters were readily hydrolyzed to a series of liquids of increasing boiling points corresponding to 2, 3–butanediol and polymers of this compound. In other words, boric acid catalyzed the condensation of two or more molecules of 2, 3–butanediol by condensing with the diol to yield an ester, two or more molecules of which condensed to form a new boric ester which was then hydrolyzed to boric acid and a polymer containing eight, twelve or more carbon atoms. This process is clearly analogous to the polymerization of glucose to starch *via* glucose–1–phosphate.

According to Silverman and Werkman (1941)[5] 2, 3–butanediol is probably produced in the bacterium by reduction of acetyl methyl carbinol, which is formed from pyruvate in the presence of phosphate.

[5] Silverman, M. and Werkman, C. H. (1941): J. biol. Chem., 138, p. 35.

CYSTINE, METHIONINE AND SULPHATE EXCRETION IN CYSTINURIA

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Recent experiments have shown that there may be a considerable variation in the response of cystinuric individuals to the ingestion of sulphur-containing amino acids. All cystinurics so far recorded have shown an increased excretion of cystine when the protein intake was increased but not when extra cystine itself was ingested. Lewis, Brown and White (1936) and Brand, Cahill and Harris (1935) have both reported cases in which methionine and cysteine hydrochloride have increased the cystine excretion. Lough *et al.* (1941) found that one cystinuric woman excreted extra cystine after taking methionine only when the protein intake was low. A pre-adolescent boy examined by Andrews and Randall (1935) did not respond in this way to methionine. When, however, he was re-examined several years later (Andrews, Andrews and Runtenber, 1938; Andrews and Andrews, 1939) it was found that an increase in urinary cystine did follow the ingestion of this amino acid. More recently Hess and Sullivan (1942) have reported a case in which neither cysteine hydrochloride nor methionine affected the cystine excretion. Another cystinuric studied by them at the same time responded not only to methionine and cysteine, but also to alanine and, to a lesser extent, to glycine and glutamic acid.

Because of this variation in the response to different amino acids it seems advisable to examine as wide a range of subjects and conditions as possible. This paper gives the results obtained with a cystinuric woman.

EXPERIMENTAL DETAILS.

The subject of these studies was a young woman, 23 years of age, who in July, 1944, had her left kidney removed because of the presence of a cystine calculus in the renal pelvis. Specimens of urine collected a fortnight after the operation were acid in reaction and were loaded with cystine crystals. The patient was then given alkalis until the urine remained markedly alkaline, and this therapy was continued throughout the experiments reported here. It was found that when the pH fell below 7·6 crystals were invariably present.

Diet.

The patient was put on a meat-free diet which contained each day the same number of eggs and the same amount of milk but was unrestricted in cereals, fruit and vegetables. The total protein intake was approximately 0·9 gm. per kilo. As the patient was boarding it was found impossible to impose more rigid restrictions.

The amino acids, cystine, methionine and cysteine hydrochloride, were taken in gelatin capsules in such amounts that the intake each day corresponded in sulphur content to 2 gm. cystine. The l-cystine used on August 24th and 25th and the dl-methionine were made available through the courtesy of Mr. H. R. Marston. The cystine used on November 15th and 16th was isolated from sheep's wool and cysteine hydrochloride was obtained from part of the same preparation by reduction with tin and hydrochloric acid.

On those days when the protein was derived from a different source (October 25, 26, 31, November 1, 21 and 22) it was given in amounts which corresponded in nitrogen content to the eggs which it replaced, i.e. 14 gm. gelatin or 3 oz. lean veal replaced 2 eggs.

During the experiment beginning on November 21 the protein intake was increased by including one serve of meat (about 3½ oz.) in the usual diet, making the total protein intake about 1·2 gm. per kilo.

Method.

The 24 hour specimens of urine were preserved under toluene. The total nitrogen was deterbined by the usual modified Kjeldahl method and the total sulphate gravimetrically (Folin). A comparator was used for measuring the pH.

The Lugg modification of the Sullivan procedure (Lugg, 1933) was used to determine the cystine content of the urine after it had been standing for three days, but otherwise no effort was made to include the "bound cystine" which has been reported by Hess and Sullivan (1942) and Sullivan and Hess (1936) to be present in the urine of cystinurics. When cystine crystals were present the urine was centrifuged, the deposit dissolved in 3 p.c. HCl and the cystine content determined separately. Results given in the Tables include both the cystine present in solution and that obtained as crystals.

Methionine was determined by the method described by Albanese *et al.* (1944), except that as most of the 24 hour specimens of urine were of rather low volume the estimations were done directly on the acidified samples without previous concentration. The estimations were done in duplicate and gave consistent results. In view of the possible interference by cystine present in the urine of this patient a series of determinations was carried out on samples to which extra cystine had been added. It was concluded that cystine did not interfere with the determination of methionine in urine under the conditions of the experiment.

Unfortunately details of this method of estimating methionine were not available until 4 months after the urine samples had been collected. During this period the specimens, which had a pH of 7·6–8·0, and had had toluene added, were kept at 4° C. In view of the results there seems no reason to believe that there was any considerable loss of methionine during this period. The daily methionine excretion was found to be of the same order as that obtained from a number of normal individuals. Twenty-four estimations on urine from twelve adults gave results varying from 250 to 640 mg. methionine per day, the majority lying between 300 and 450 (compare Albanese *et al.*, 1944, who found in seven normal individuals a range of 247 to 494 mg. methionine excreted each day). Two specimens of normal urine allowed to stand under similar circumstances for 8 weeks showed no loss of methionine.

RESULTS AND DISCUSSION.

The results are shown in Tables 1, 2 and 3 and Fig. 1. The excretions of extra cystine, methionine and sulphate following the ingestion of certain amino acids and proteins were calculated for the two days on which they were fed and the following day. Normal values were obtained from the two, or where possible, three days preceding the test period.

Cystine.

When cystine was fed to the patient the effect was similar to that obtained with other cystinurics, that is, there was a rise in urinary sulphate during the following 24 hours but no increase in cystine. In fact, on both occasions, there was a slight fall in cystine excretion which was not accompanied by a corresponding fall in nitrogen excretion (Fig. 1). The per cent. of cystine recovered as sulphate was lower than that obtained by the majority of workers. The most probable explanation of this is that, the protein intake being unusually low, a high proportion of ingested cystine was used for tissue replacement. As others have found, the urinary sulphate excretion had returned to normal 24 hours after the ingestion of this amino acid.

Methionine.

The increase in urinary excretion of sulphate and of cystine which followed the ingestion of methionine was similar to the response obtained from the majority of cystinurics, though the per cent. recovery as cystine was higher and as sulphate was lower than usual Lough *et al.* (1941) and Lewis *et al.* (1936) who used similar doses of

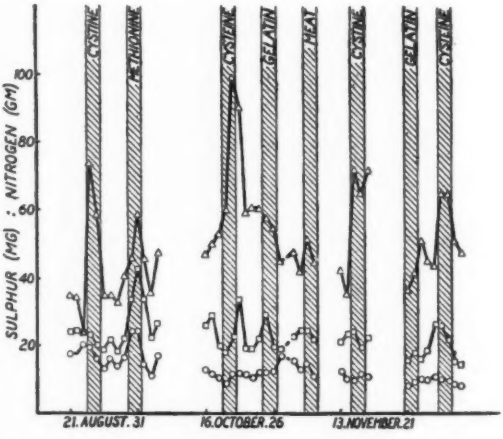

Fig 1. Ratio of urinary sulphate, cystine and methionine sulphur to total urinary nitrogen excreted each day.

△ Sulphate sulphur (mg.) : Total nitrogen (gm.).

□ Cystine sulphur (mg.) : Total nitrogen (gm.).

O Methionine sulphur (mg.) : Total nitrogen (gm.).

methionine found that less of it appeared as cystine when the protein intake was high (about 1·9 gm./kilo.) than when it was moderate (1·2 gm./kilo.). Brand, Cahill and Harris (1935) who studied a cystinuric on a lower protein diet (average nitrogen excretion 7·0 gm./day) recovered about 14 p.c. methionine as cystine. The large proportion of methionine obtained as cystine in this experiment can therefore be attributed largely to the small amount of protein consumed at this time (urinary N. 4·8 gm./day).

TABLE 1.

Influence of protein and of S-containing amino acids on urinary sulphur in cystinuria.

Date.	Substance fed.	Volume. ml.	Total N. gm.	Sulphur (gm.). Cystine.	Methionine.	Sulphate.
Aug. 21*		1,300	6·1	0·15	0·11	0·21
„ 22*		1,650	4·7	0·12	0·08	0·16
„ 23*		1,200	2·9	0·07	0·06	0·07
„ 24	2 gm. cystine	1,870	5·2	0·12	0·10	0·38
„ 25	„ „	1,200	3·7	0·07	0·06	0·22
„ 26		1,750	5·3	0·10	0·07	0·18
„ 27*		1,320	4·4	0·10	0·07	0·15
„ 28*		1,575	5·6	0·10	0·08	0·19
„ 29*		1,870	4·6	0·10	0·08	0·19
„ 30	2·5 gm. methionine	1,770	4·6	0·14	0·12	0·21
„ 31	„ „	1,980	6·2	0·27	0·15	0·37
Sept. 1		1,850	5·0	0·17	0·07	0·23
„ 2		1,100	4·2	0·09	0·05	0·10
„ 3		1,470	4·7	0·13	0·08	0·22
Oct. 16†		1,040	5·2	0·14	0·07	0·25
„ 17†		700	4·8	0·14	0·06	0·25
„ 18†		550	4·7	0·10	0·05	0·25
„ 19	2·7 gm. cysteine hydrochloride	600	5·4	0·10	0·05	0·33
„ 20	„ „ „	750	5·2	0·12	0·06	0·52
„ 21		1,050	5·4	0·18	0·06	0·50
„ 22†		720	5·4	0·10	0·06	0·32
„ 23†		1,200	6·2	0·12	0·07	0·37
„ 24†		610	4·6	0·10	0·06	0·28
„ 25	14 gm. gelatin replaced 2 eggs	1,090	5·4	0·16	0·07	0·31
„ 26	„ „ „ „	810	5·1	0·10	0·06	0·28
„ 27		810	5·2	0·10	0·09	0·23
„ 28		No specimen collected.				
„ 29†		1,140	5·7	0·16	0·09	0·27
„ 30†		700	5·3	0·13	0·07	0·22
„ 31	3 oz. veal replaced 2 eggs	670	5·0	0·13	0·08	0·26
Nov. 1	„ „ „ „	950	6·0	0·13	0·07	0·27
„ 13		1,300	7·0	0·14	0·09	0·31
„ 14		1,270	5·0	0·12	0·05	0·16
„ 15	2 gm. cystine	720	5·3	0·13	0·05	0·38
„ 16	„ „	730	5·0	0·10	0·06	0·33
„ 17		600	3·4	0·08	0·04	0·25
„ 21	14 gm. gelatin replaced 2 eggs	880	8·2	0·14	0·07	0·30
„ 22	„ „ „ „	1,100	8·5	0·16	0·08	0·35
„ 23‡		900	6·8	0·10	0·07	0·36
„ 24‡		800	7·4	0·14	0·07	0·33
„ 25‡		800	4·5	0·12	0·05	0·20
„ 26	2·7 gm. cysteine hydrochloride	1,350	10·0	0·26	0·10	0·65
„ 27	„ „ „	800	6·8	0·16	0·07	0·44
„ 28		1,230	9·7	0·15	0·08	0·50
„ 29‡		1,100	9·2	0·13	0·08	0·44

* These values used in calculating average values for low protein diet period 1. (Table 3.)
† These values used in calculating average values for low protein diet period 2. (Table 3.)
‡ These values used in calculating average values for medium protein diet. (Table 3.)

Recently experimental evidence of the way in which methionine is broken down to cysteine has been obtained. Binkley and du Vigneaud (1942) observed that when dl. homocysteine and dl. serine were shaken with rat liver slices l-cysteine was formed while Stetten (1942) has shown that cystine derived from rats fed with serine labelled with N^{15} had an unusually high proportion of the isotope. Since the inborn error in the metabolism of cystinurics is in handling cysteine the amount of extra urinary cystine derived from methionine would depend upon the availability of serine and methionine, the rate of demethylation and the rate of conversion of homocysteine to cysteine. When the protein intake is low the cystine excretion may be limited by lack of available serine and methionine, but when the protein intake is high the rate of conversion of homocysteine to cysteine may already be at its maximum so that the ingestion of extra methionine cannot increase cysteine production and this amino acid is disposed of by some other mechanism. This would mean that at high protein levels neither homocysteine nor methionine would produce extra cysteine (as was found by Lough and colleagues, 1939) and that equivalent amounts of methionine and homocysteine should give similar increases in the urinary cystine excretion at lower protein levels as was found by Brand, Cahill and Block (1935).

Information about the excretion of methionine by normal individuals is scanty. Values obtained throughout these experiments were of the same order as those found by Albanese *et al.* (1944) for normal individuals, and were within the range found for twelve adults whose urine was tested during these experiments. Andrews and Randall (1935) using a method involving the recovery of methyl iodide found that both a normal and a cystinuric boy excreted 20–40 mg. methionine sulphur per day and that in both cases the administration of 2·33 gm. dl-methionine caused an excretion of 10–15 p.c. of the ingested methionine. The recovery of methionine in these experiments lay within the same range, though the normal urinary methionine was higher. It cannot be assumed that the undetermined organic sulphur fraction as reported by most other workers is largely due to methionine, as the Benedict-Denis method of determining total sulphur (which is usually used) is known to give unreliable results when methionine is present (Masters, 1939).

As would be expected there was an immediate rise in methionine excretion after the administration of this amino acid, followed by a slower rise in cystine and sulphate excretion. This no doubt reflects the blood level of these constituents. Brown and Lewis (1941) found that in normal rabbits the ingestion of methionine was followed by a rise in plasma cystine and that the organic sulphur rose to a higher level for longer periods than after an equivalent amount of cystine, while the increase in sulphate content was greater and occurred more rapidly after cystine.

TABLE 2.

Extra sulphur excreted after cystine, methionine and cysteine hydrochloride.

Substance fed.	Amount fed. (gm.).	Date.	Average N. excretion (gm./day).	Extra sulphur excreted as Cystine. gm.	p.c.	Methionine. gm.	p.c.	Sulphate. gm.	p.c.	P.c. amino acid recovered.
Cystine	4	Aug. 24–26	4·8	—0·05	—5	—0·02	—2	0·34	32	32
„	„	Nov. 15–17	5·2	—0·09	—8	—0·06	—5	0·25	24	24
Methionine	5	Aug. 30–Sept. 1	4·8	0·28	26	0·11	10	0·28	26	62
Cysteine hydrochloride	5·4	Oct. 19–21	5·3	0·02	2	0	0	0·60	56	58
„	5·4	Nov. 26–28	8·8	0·21	20	0·06	5	0·70	66	91

Cysteine Hydrochloride.

In contrast to cystine the ingestion of cysteine hydrochloride was followed by a rise in cystine which accounted for 1 p.c. ingested compound when the protein intake was low, and 19 p.c. when the protein intake was raised to 1·2 gm./kilo. This variation could be attributed to the very low protein content of the diet during the earlier experiment assuming that at this level relatively more cysteine can be used as such. A higher proportion of the cysteine hydrochloride appeared as sulphate than occurred after either cystine or methionine, and more was excreted when the protein intake was raised. However, only in the latter case did the total recovery as cystine and sulphate approach values obtained by other workers (80–98 p.c.). This can also be attributed to greater utilization of cysteine as such when there is little protein in the diet.

An enzyme capable of converting cysteine to H_2S, pyruvic acid and ammonia has been isolated from the liver of rats (Laurence and Smythe, 1943) and mice (Binkley, 1943) which resembles and may be identical with that which produces pyruvic acid from serine. It is conceivable that in the cystinuric there is a deficiency of this enzyme so that excess cysteine accumu-

lates and appears in the urine as cystine. At the same time the failure of the enzyme to remove extra serine might be responsible for the preferential oxidation of methionine to cysteine.

In these experiments at a moderate level of protein intake (though not at a low level) the ingestion of cysteine hydrochloride caused a small rise in methionine excretion, but this was accompanied by an equivalent rise in urinary nitrogen.

Replacement of Eggs by Gelatin or Meat.

In order to reduce the concentration of cystine in the urine of cystinurics it is usual to advise the consumption of a diet low in protein, more particularly in animal protein which contains on the whole, rather more cystine and methionine than that of vegetable origin. It was therefore of interest to see whether the substitution of proteins containing different proportions of the sulphur-containing amino acids would affect the urinary concentration of cystine appreciably. The two eggs in the basal diet were replaced on two consecutive days by either gelatin or muscle meat containing an equivalent amount of protein. Assuming that these foodstuffs had the following amino-acid composition (Block and Bolling, 1944):

	Cystine.	Methionine.
Eggs	2·1 p.c.	4·0 p.c.
Meat	1·1 p.c.	3·3 p.c.
Gelatin	0·1 p.c.	0·8 p.c.

then, when meat replaced eggs, there would be a reduction in cystine intake of the order of 150 mg./day, and of methionine of 170 mg./day; and when gelatin replaced eggs the reduction would be about 230 mg. cystine and 340 mg. methionine.

An examination of the results shows that at this level of protein intake the use of protein with a lower content of sulphur-containing amino acids did not reduce either the cystine, methionine or sulphate excretion appreciably, and, in fact, there was a slight rise in cystine excretion after the gelatin. This suggests that either the protein intake was so low that the extra cystine and methionine which would be derived from the eggs (provided it was not taken in large amounts over a short period, as after test doses of the amino acids) was required and completely utilized by the tissues, or that cystine excretion is influenced more by the amount than the kind of protein consumed. Hess and Sullivan (1942), for example, found that in one cystinuric the ingestion of alanine and to a lesser extent of glycine and glutamic acid caused a rise in cystine excretion. Possibly a similar effect was obtained in this case so that a lower intake of S-containing amino acids was counteracted by the increased excretion caused by the glycine present in the gelatin. It should, however, be remembered that the expected difference would be very small if (as occurred when the pure amino acid was fed) only about 24 p.c. cystine and 26 p.c. methionine were recovered as sulphate, 26 p.c. methionine as cystine and 10 p.c. as methionine. The expected values for sulphur excretion obtained by calculation and those found by experiment are shown in Table 3. Both on theoretical and on practical grounds the restriction in the consumption of proteins rich in S-containing amino acids (at this level of protein intake) would be unwarranted in this cystinuric as a means of reducing the excretion of cystine.

TABLE 3.

Expected and observed differences in sulphur excretion with variations in protein intake.

			Av. S (gm./day) excreted as			Expected S (gm./day) excretion as		
Protein level.	Date.	Average N. excretion (gm./day).	Cystine.	Methionine.	Sulphate.	Cystine.	Methionine.	Sulphate.
Low (1)	Aug.*	4·8	0·11	0·08	0·16			
Low (2)	Oct.†	5·2	0·12	0·07	0·27			
Medium	Nov.‡	7·0	0·12	0·07	0·33	0·14	0·08	0·30
Gelatin replaced eggs	Oct. 25–26	5·2	0·12	0·07	0·28	0·10	0·06	0·23
„ „ „	Nov. 21–22	7·8	0·15	0·07	0·33	0·10	0·06	0·29
Meat replaced eggs	Oct 31–Nov. 1	5·5	0·13	0·07	0·27	0·11	0·07	0·25

*, †, ‡ See Table 1.

Affect of Different Levels of Protein Intake.

Throughout these experiments the nitrogen excretion was sometimes rather irregular and occasionally fell to values which were less than would be expected even from a fasting individual. In most cases this seems to have been due to a failure in the collection of an exact 24 hour specimen of urine as the reduction in nitrogen content was usually associated with a fall in the other constituents too, and low values were followed by ones rather higher than normal on the succeeding day. To overcome this difficulty the results have been expressed as cystine, methionine and sulphate sulphur per unit of nitrogen excreted (Fig. 1). This presentation of the results also shows the relative efficiency of the metabolism of the S-containing amino acids under different circumstances.

Although the amount of protein consumed remained the same, the nitrogen excretion during October and up to November 17 was higher than that during August and September. Not only did the total nitrogen increase, but the sulphate excretion was about 60 p.c. higher during the second period, while the methionine and cystine excretion remained about the same. A possible interpretation of this is that in August when the patient was still convalescing after the removal of one kidney, there was considerable retention of protein and S-containing amino acids (presumably chiefly cystine) for tissue repair.

When the protein intake was raised by including about 3½ oz. meat in the usual diet, an increase in cystine, methionine and sulphate excretion would be anticipated. In practice it was found that urinary sulphate was higher, and the cystine and methionine lower than would be expected (Table 3). Other investigators (Lewis and co-workers, 1936; Lough and co-workers, 1941) have drawn attention to the fact that less cystine is excreted after the ingestion of methionine at a high than at a moderate level of protein intake and attribute this to better utilization. The noticeable reduction in the ratio of urinary methionine to nitrogen and, to a smaller extent, of urinary cystine to nitrogen (Fig. 1) found when the protein intake was raised from 0·9 gm./kilo. to 1·2 gm./kilo. gives more conclusive evidence of this increased efficiency in the utilization of methionine.

SUMMARY.

Total nitrogen and cystine, methionine and sulphate sulphur have been determined in the urine of a cystinuric woman.

When cystine was fed no extra cystine or methionine was excreted, but there was a marked rise in urinary sulphate during the succeeding 24 hours.

When methionine was fed there was an immediate rise in methionine excretion followed by a slower rise in cystine and sulphate excretion which continued for two days.

When cysteine hydrochloride was fed the methionine excretion remained constant, but there was an increase in urinary cystine and sulphate which was more marked when the protein intake was raised from 0·9 to 1·2 gm./kilo.

The p.c. recovery of cystine, methionine and cysteine as sulphur in the urine was much less than in other cystinurics. This is believed to be due to the small amount of protein consumed during these experiments.

When the protein intake was increased from 0·9 to 1·2 gm./kilo there was a rise in the excretion of cystine, methionine and sulphate excretion. The proportion of cystine and methionine excreted per unit of nitrogen was less, and that of sulphate sulphur more at the higher than the lower level of protein intake.

Two levels of excretion were found when the patient was receiving 0·9 gm. protein/kilo. One level was determined shortly after the patient had undergone a severe surgical operation and showed a markedly lower excretion of nitrogen and sulphate sulphur.

When the two eggs in the basal diet were replaced by an equivalent amount of gelatin the expected fall in cystine, methionine and sulphate excretion did not take place.

The replacement of the eggs by meat was not accompanied by a fall in sulphur excretion.

The use of plant proteins in preference to those of animal origin as a means of reducing the cystine excretion in this patient is considered unwarranted when the protein consumption is low or moderate.

Possible interpretations of these results are discussed.

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THE DETERMINATION OF VITAMIN C BY THE GROWTH OF GUINEA-PIGS

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The administration of antiscorbutic foodstuffs to young guinea-pigs living on a scorbutic diet, has been observed to evoke growth responses which are dependent on the amount of vitamin C in the supplements fed. Bracewell, Hoyle and Zilva (1930) used the weight increase of guinea-pigs as one criterion in an assessment, by bioassay, of the vitamin C in apples. The methods of measurement which have been elaborated by Coward and Kassner (1936) and Coward (1938) and by Harris and Olliver (1942) depend solely on the growth response of young guinea-pigs to graded intakes of vitamin C. The former used a preventive method in which antiscorbutic supplements were fed from the beginning of the experiment. In the curative method of Harris and Olliver there was an initial period of about ten days when the experimental animals received only the scorbutic diet. These methods, with modifications of the basal diet and of the duration of the experiment have been used by numerous investigators (Gould, Tressler and King, 1936; Mack, Tressler and King, 1936; Tressler, Mack and King, 1936; Harris and Poland, 1937; Tressler, Mack, Jenkins and King, 1937; Fenton, Tressler, Camp and King, 1938; Mack, Tapley and King, 1939; Wheeler, Tressler and King, 1939; Kirk and Tressler, 1941; Dunker, Fellers and Esselen, 1942; Cruickshank, 1943).

The procedure followed in preliminary experiments carried out by the growth method in this laboratory was based on conclusions which were drawn from published data. Only two levels of intake of the vitamin were employed, namely 0·4 mg. and 0·8 mg. ascorbic acid per day. From the experience of others it was expected that a significant difference in the growth response of guinea-pigs to these amounts of vitamin C would result, without entailing a degree of depletion on the lower level of intake so severe as to cause actual loss in weight.

Groups of five guinea-pigs, subsisting on a basal diet supposedly devoid of vitamin C, were given each day supplements containing, on the basis of chemical analyses, the equivalents of 0·4 mg. and 0·8 mg. ascorbic acid. These were administered for 6 weeks. The response was estimated by the curative and by the preventive methods.

In each case the difference between the mean weight increments of groups of guinea-pigs ingesting respectively 0·4 mg. and 0·8 mg. of pure ascorbic acid per day, was so small as to be statistically insignificant. The five negative control animals on the basal diet all developed scurvy and died in 23, 24, 24, 25 and 31 days respectively. (The negative control guinea-pigs weighed from 200 to 280 gm. at the beginning of the experiments).

The response to the supplements was variable and the failure to secure a significant difference between the mean weight increments of the groups of guinea-pigs on the two levels of ascorbic acid intake was probably due to the small number of animals employed. Nevertheless, the good growth of those which received only 0·4 mg. ascorbic acid daily, roused the suspicion that the

basal diet might contain traces of vitamin C. It was that used by Coward and Kassner (1936) and was composed of:

	p.c.
Bran	45
Crushed oats	25
Dried skimmed milk	30

Vitamins A and D were supplied as a concentrate of halibut liver oil administered separately. The powdered milk was heated in shallow dishes for 2 hours at approximately 105–110° C.

Since there was no trace of reducing material in the bran or oats, they were assumed to be devoid of vitamin C. Extracts of the heated milk powder on the other hand were observed to reduce 2, 6–dichlorphenolindophenol. The nature of these reducing substances is discussed later. Their presence emphasized the possibility that the basal diet might contain traces of vitamin C. In consequence a study of basal diets which contained no milk was undertaken.

An investigation was made of the capacity of the following diets, when supplemented with ample vitamin C, to provide for the requirements for normal growth of guinea-pigs.

Diet 3 was designed to contain the same amount of milk protein as that of Coward and Kassner (1936). It consisted of:

	p.c.
Bran	29·0
Pollard	29·0
Oatmeal	32·3
Casein (B.D.H. Light White)	8·0
$CaCO_3$	0·7
NaCl	1·0

Pollard was introduced to enable the casein to be mixed satisfactorily with the other ingredients. Without it the casein separated from the coarser bran and oatmeal. The pollard contained a just discernible trace of reducing material. However, this proved to be a slowly reducing substance and not vitamin C (Bessey, 1938).

Diet 4 was based on that of Harris and Olliver (1942) though not identical with it. It had the following composition:

	p.c.
Bran	20·5
Pollard	20·5
Oatmeal	41·0
Fresh egg yolk	16·0 (wet wt.)
$CaCO_3$	1·0
NaCl	1·0

The dry ingredients were mixed and part was added to the beaten egg yolk. This mixture was made into biscuits and baked. It was then minced and added to the rest of the diet. After some weeks the method of preparation was changed. Eggs were hard boiled and the separated yolks mixed into the diet without further cooking.

Diet 5 was made up to contain:

	p.c.
Bran	22
Pollard	22
Oatmeal	44
Dried yeast	10
$CaCO_3$	1
NaCl	1

Guinea-pigs bred in the laboratory were weaned and when their body weight was 300 gm. they were transferred from the stock diet to one of the above diets. They were given 5 mg. pure ascorbic acid in 0·06 p.c. acetic acid by a pipette on six days each week. To ensure a regular supply of B vitamins, in case of decline in appetite, a yeast extract was given to the animals. This was prepared by boiling moist compressed bakers' yeast for 5 minutes with twice its weight of 0·02 p.c. acetic acid and filtering. 2 ml. of this extract were administered by pipette on six days each week. Some of the animals were also given ferrous sulphate in solution, equivalent to 1 mg. Fe six times weekly. They all received a concentrate of halibut liver oil. The supplements were administered at different times of the day. The experiment was continued till the guinea-pigs were nineteen weeks of age.

The growth of all the guinea-pigs on diet 4 (four males and three females) was definitely inferior to that of the stock animals. The same was true for the three animals (males) on diet 5. These diets were, therefore, discarded as inadequate. The growth on diet 3 was not quite equal

to that on the stock diet as shown in Table 1. The stock diet had the following percentage composition: ground maize 11, crushed wheat 15, pollard 19, oatmeal 30, liquid milk 24, common salt 0·3 and cod liver oil 0·6. Fresh lucerne was fed *ad lib.*

As a result of this experiment, diet 3 was considered to be satisfactory as a basal diet for vitamin C investigations.

TABLE 1.

Weight of guinea-pigs at different ages on diet 3 and on the stock diet.

Diet.	Weight of guinea-pigs. Age 10 weeks. Male.	Age 10 weeks. Female.	Age 15 weeks. Male.	Age 15 weeks. Female.	Age 19 weeks. Male.	Age 19 weeks. Female.
	gm.	gm.	gm.	gm.	gm.	gm.
3	390*	375*	480*	490*	512*	575*
	420	395*	510	465*	565	512*
	473	425*	646	535*	730	597*
	515	430	660	508	737	553
Mean	450	406	574	499	636	559
Stock mean	418	367	577	491	657	568

* These animals received a supplement of 1 mg. Fe on six days each week.

Comparison of Basal Diets with and without Milk Powder.

Diet 3 was compared with the diet previously used, in an experiment designed to find out if the earlier observations on the growth of guinea-pigs would be repeated on a diet known to be devoid of vitamin C.

Guinea-pigs were weaned at 250 gm. weight on to one or other of the basal diets plus greenfeed. Half the animals received diet 3 without iron, the other half was fed on the basal diet of Coward and Kassner (1936). The dried skimmed milk was heated for 4 hours but at a lower temperature than in the earlier experiments to prevent undue caramelization. During heating it set into cakes. To break up these lumps and to ensure thorough exposure to air the milk was twice removed from the oven during the 4 hour period, for stirring and sieving. This diet is referred to as diet 1 or the milk diet.[1]

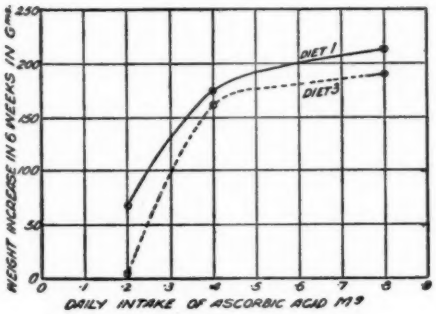

Fig. 1. Mean weight increments of groups of guinea-pigs in an experimental period of six weeks, as influenced by the ascorbic acid intake.

Upper curve: Basal diet 1.
Lower curve: Basal diet 3.

Both male and female animals were used and so far as possible litter mates were compared on the two diets. From the time of weaning at 250 gm. each animal received 10 gm. fresh lucerne daily till a weight of 300 gm. was reached when the lucerne was withdrawn and the vitamin C supplements fed immediately. Halibut liver oil concentrate and yeast extract were administered to all guinea-pigs. There were three negative controls on each diet. The other animals received 0·2, 0·4 or 0·8 mg. ascorbic acid daily for 6 weeks. For the following 3 weeks the vitamin C intake of the guinea-pigs receiving 0·4 or 0·8 mg. was increased to 5 mg. This was done to assess the growth promoting capacity of the diets when supplemented with adequate vitamin C and to show to what extent growth had previously been restricted by the vitamin C intake. Finally, growth was measured for a period of three weeks on the stock diet with greenfeed *ad lib.*

Results.

The most important results of this experiment are summarized in Tables 2 and 3. The data for the female animals, only two or three in a group, have been omitted to save space. The weight increments after 6 weeks are shown graphically in Fig. 1.

[1] In this experiment oatmeal was used instead of crushed oats.

TABLE 2.

Weight increase of guinea-pigs on diets 1 and 3 supplemented with ascorbic acid.

Diet.	Daily intake of ascorbic acid. mg.	Number of male animals.	Mean weight increase. In 4 weeks. gm.	S.D. of mean. gm.	In 6 weeks. gm.	S.D. of mean. gm.
1	0·2	4	105	11	68	19
3	0·2	4	43	11	3	10
1	0·4	5	118	8	174	13
3	0·4	6	117	7	161	10
1	0·8	4	147	10	214	11
3	0·8	5	141	5	190	7

TABLE 3.

Weight increase of guinea-pigs on diets 1 and 3 supplemented with ascorbic acid, compared with growth on the stock diet.

Diet.	Daily intake of ascorbic acid during 6 weeks. mg.	Number of male animals.	Age at 300 gm. days.	Mean weekly weight increments.* Weeks 1–6. gm.	Weeks 7–9. gm.	Weeks 10–12. gm.
1	0·4	5	42	29	21	36
3	0·4	6	44	27	22	26
1	0·8	4	43	36	28	29
3	0·8	5	40	32	23	29
Stock		8	47	36	23	21

* The animals were fed on the experimental diets for 9 weeks and then on the stock diet for 3 weeks. During the first 6 weeks the diets were supplemented with 0·4 mg. or 0·8 mg. ascorbic acid daily, during the next 3 weeks with 5 mg. ascorbic acid daily.

There were three negative control animals in each group, all females. Those on diet 1 died after 37, 36 and 31 days (mean 35) without vitamin C, on diet 3 after 28, 27 and 30 days (mean 28). Analysis of the variance of the weight increments observed in the experimental animals led to the following conclusions.

1. The difference between the mean weight increments of the guinea-pigs on diets 1 and 3 was not significant at either the 0·4 mg. or the 0·8 mg. level of ascorbic acid intake. Thus:

Difference between mean weight increments on 2 diets.			Level of significance of difference.
0·4 mg. level (11 animals)	4 weeks	1 gm.	$P > 0·9$
	6 weeks	13 gm.	$P = 0·6$
0·8 mg. level (9 animals)	4 weeks	6 gm.	$P = 0·7$
	6 weeks	24 gm.	$P = 0·25$

2. The difference between the mean weight increments of the animals ingesting 0·4 mg. and 0·8 mg. ascorbic acid respectively was no more significant on diet 3 than on diet 1. The differences were:

Difference between mean weight increments on 2 levels of intake.			Level of significance of difference.
Diet 1 (9 animals)	4 weeks	29 gm.	$P = 0·18$
	6 weeks	40 gm.	$P = 0·18$
Diet 3 (11 animals)	4 weeks	24 gm.	$P = 0·12$
	6 weeks	29 gm.	$P = 0·17$

3. The growth of the guinea-pigs receiving 0·2 mg. ascorbic acid per day was greater on diet 1 than on diet 3. The level of significance of the difference was $P = 0·04$ (4 weeks), $P = 0·09$ (6 weeks).

4. The negative control animals lived longer on diet 1 than on diet 3. The level of significance of the difference was $P = 0·04$.

Nature of the Reducing Material in Heated Milk.

Studies of some of the reducing substances in liquid and dried milk have been carried out, mainly by investigators who have been interested in the development of "oxidized" and "cooked" flavours in heated milk. No attempt will be made to review this literature here (Gould and Sommer, 1939; Fleisch and Domenjoz, 1940; Gjessing and Trout, 1940; Gould, 1940; Stamberg and Bailey, 1943; Townley and Gould, 1943; Harland and Ashworth, 1945).

Experimental. A batch of dried skimmed milk used in an earlier experiment was examined for indophenol-reducing substances before and after heating for 2 hours at 105–110° C. Indophenol titration of a 3 p.c. metaphosphoric acid extract, without treatment with H_2S, indicated a seven-fold increase in reducing capacity as a result of heating. The total ascorbic acid content estimated by the method of Lugg (1942), after treatment with H_2S, was approximately 1·4 mg. p.c. in both unheated and heated milk.

A sample of milk used in the latter part of the experiment discussed in the next communication was also examined. An extract was prepared by the method of Woessner, Elvehjem and Schuette (1939). The extract of the dried skimmed milk before heating contained no material capable of reducing the indophenol dye solution. Nevertheless, after 4 hours' heating, the reducing capacity, without H_2S treatment, was equivalent to 10 mg. of ascorbic acid per 100 gm. of milk powder. The reducing material gradually disappeared on standing in the acid extracting solution and it could not be stabilized by displacing the air in the vessel with CO_2. In this it differed from the reducing substances in processed foods examined by Wokes, Organ, Duncan and Jacoby (1943).

When examined by Lugg's method for the determination of reduced ascorbic acid, the reducing substance generated in the milk by heating was found to condense completely with formaldehyde at pH 3·5 and about half of it condensed with formaldehyde at pH 1·5 under the conditions prescribed for carrying out the titrations. By this method, therefore, about 50 p.c. of it would appear to consist of ascorbic acid. The method of Levy (1943) was also employed. An extract of heated milk was titrated against the dye solution before and after the addition of concentrated HCl. Accurate titration of the small amount of reducing material in the milk extract was not possible in the presence of HCl. The most that can be said is that less than half would appear to be ascorbic acid according to Levy's technique.

DISCUSSION.

In the measurement of vitamin C by bioassay the experimental animals are commonly fed on a basal diet containing heated skimmed milk powder. When such a diet was fed to guinea-pigs in this laboratory the growth of the animals receiving a supplement of 0·4 mg. ascorbic acid daily was not significantly less than the growth of those receiving twice this amount. The possibility that additional vitamin C was being ingested from the milk was therefore considered.

A comparison was made of the growth of guinea-pigs on the diet containing milk with that on a diet without milk and shown by chemical tests to be devoid of vitamin C. The results of this experiment are set out above.

The substitution of a basal diet known to be devoid of vitamin C, for one suspected of containing it, did not result in an increased difference between the weight increments of the guinea-pigs ingesting 0·4 mg. and 0·8 mg. ascorbic acid, respectively. Nor was the growth of the animals in either of these groups significantly less on the new diet than on the old. It is therefore not necessary to postulate the presence of vitamin C in the milk diet to explain the growth phenomena previously observed.

Nevertheless the experiment did not prove that this diet was free from vitamin C. The negative control guinea-pigs lived longer and those on the 0·2 mg. level of ascorbic acid intake grew better when subsisting on the milk diet than on that containing casein. Had the powdered milk contained approximately 1 mg. p.c. of ascorbic acid these results might have been expected. On the higher levels of intake the difference between the animals on the two diets was much less marked because their requirements for vitamin C were almost met from the ascorbic acid supplements.

However, though the contamination of the diet with vitamin C is the most obvious, it is not the only explanation which may be put forward to account

for the superiority, on the lower levels of intake, of the guinea-pigs ingesting milk. The physical condition of seriously depleted animals is, in all probability, partly dependent on factors which are only indirectly related to the intake of vitamin C. McHenry, Reedman and Sheppard (1938) have shown that part of the loss in weight which occurs in scurvy is due specifically to a deficiency of vitamin C. It is possible that part is due to a falling off in food consumption traceable to the toothache occurring in the scorbutic condition. It may be that in the present experiment the milk diet was more readily eaten by the scorbutic animals than diet 3, or that it contained some essential nutrient in greater abundance. When food consumption declined this might become a limiting factor in the diet. Some evidence for this suggestion may be put forward.

The decline in weight of the negative control animals in the above experiment began after the 11th day. The mean weight increments of the male animals up to this time were:

Diet 1.		Diet 3.	
Intake of ascorbic acid.	Wt. increase in 11 days.	Intake of ascorbic acid.	Wt. increase in 11 days.
mg.	gm.	mg.	gm.
0·2	49	0·2	30
0·4	48	0·4	53
0·8	50	0·8	50

Weight increase during this period is thought not to depend on the level of ascorbic acid intake (Key and Elphick, 1931). This has been confirmed by an experiment carried out in this laboratory in which the preventive method and the milk diet were used. There was no correlation between the weight increase during this period and the ascorbic acid intake, the negative control animals growing as well as any other group. It follows that the difference between the mean weight increments of the groups of guinea-pigs receiving 0·2 mg. ascorbic acid must be due to some other factor. The animals in the two groups were as similar as possible. There were three pairs of litter mates and the mean age at 300 gm. weight of the four animals in each group was 49 days and 47 days on diets 1 and 3 respectively. The factor which was responsible for the difference in these animals at 11 days may also have been the cause of the difference after 4 and 6 weeks. If this possibility is admitted it must be concluded that at the level of intake of vitamin C where the dose-response curve rises most steeply, factors other than vitamin C may influence the weight of the animals. Coward (1938) has already pointed out that the principal disadvantage of the growth method is its lack of specificity.

The death of the negative control guinea-pigs is commonly supposed to indicate that the basal diet is free from vitamin C, but the survival periods reported in the literature vary widely. Thus guinea-pigs whose initial body weights were from 250 to 350 gm., when confined to diets apparently devoid of vitamin C, have been found to survive for periods of 20 to 45 days (usually not more than 34 days) (Sherman, La Mer and Campbell, 1922; Zilva, 1936; Harris and Poland, 1937; Coward, 1938; Todhunter and Sparling, 1938). A small concentration of the vitamin in the diet would be expected to delay the death of the animals for only a short time, however, since once the scorbutic condition develops, food consumption declines and with it the intake of ascorbic acid. In the experiment reported above and in another similar experiment, guinea-pigs subsisting on the basal diet containing milk died in 29 to 37 days. This cannot be considered to prove that the diet was free from vitamin C.

Biological tests thus failed to determine whether the milk diet was or was not free from vitamin C. Nor could any certainty be reached as a result of chemical analyses. Dry ascorbic acid is comparatively stable: heating it for two hours at 109° C. in air caused less than 1 p.c. destruction. An attempt to determine the stability of ascorbic acid in powdered milk proved unsuccessful, since chemical examination revealed the presence in heated dried milk of substances whose reducing properties closely resembled those of ascorbic acid, and which could not be distinguished from it with certainty.

Until some reliable test is devised for the presence of vitamin C in powdered milk, its use in basal diets for the bioassay of vitamin C must be viewed with suspicion.

SUMMARY.

An investigation has been made of basal diets for use in the bioassay of vitamin C. The diet which contained heated powdered skimmed milk was suspected of containing traces of the vitamin, but biological tests by the growth method failed to determine whether any were present.

Chemical examination showed that substances closely resembling ascorbic acid in their reducing properties are produced in powdered milk by heating. Their presence prevented the determination of vitamin C in the heated milk.

In view of these facts it is considered inadvisable to include powdered milk in basal diets for the bioassay of vitamin C.

An alternative diet is suggested.

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THE VITAMIN C CONTENT OF DRIED LUCERNE *(MEDICAGO SATIVA)*; CHEMICAL AD BIOLOGICAL ASSAYS

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Chemical determinations of vitamin C in fresh plant materials, when carried out by methods based on the reducing properties of the vitamin, have been shown repeatedly to bear a direct relationship to the antiscorbutic potency as estimated by bioassays. The chemical determination of vitamin C in dehydrated foodstuffs, however, is frequently complicated by the presence of ill-defined substances such as reductones, which are formed during processing and which exhibit reducing properties similar to those of ascorbic acid. Indophenol reduction is thus not a reliable measure of the antiscorbutic potency of processed foods.

While investigating the vitamin C content of South African edible plants, Levy and Fox (1935) found that lucerne (*Medicago sativa*) was a rich source of the vitamin. They demonstrated that the ascorbic acid content of the fresh leaves as measured by indophenol titration was of a similar order to that found by a semi-quantitative bioassay in which no reference standard was used.

In a study of the vitamin C content of green leaves conducted in this laboratory, Marston, Quinlan-Watson and Dewey (1943) described methods by which the greater part of the original ascorbic acid of lucerne leaves could be retained during drying and preserved in the dehydrated material.

The bioassays reported here were undertaken as a check on the chemical methods employed in those investigations.

In preliminary experiments it was found that guinea-pigs would not eat dried powdered lucerne leaf-meal unless it was mixed with part of the basal diet. Even when this was done it was many hours before the whole of the daily ration was consumed and during this period some loss of vitamin C might occur. It was decided, therefore, to compress the lucerne meal into tablets. These were placed in the mouth of the guinea-pigs with forceps. They were promptly ground up by the molar teeth and swallowed.

MEASUREMENT OF VITAMIN C IN LUCERNE BY THE GROWTH METHOD.

Experimental.

The technique employed in the estimation of vitamin C by the growth method has been described in the preceding communication. In the present experiment guinea-pigs were weaned at 250 gm. weight on to the basal diet 1 (the milk diet[1]) already described and were given 10 gm. fresh lucerne daily. Halibut liver oil concentrate and yeast extract were fed throughout the experiment. At exactly 300 gm. weight the lucerne was withdrawn and the basal diet only fed for 9 days. On the 10th day the feeding of supplements was begun. The animals were not entirely depleted at this stage since the negative controls continued to grow for a few days longer. There were four control groups of guinea-pigs receiving 0·2, 0·3, 0·4 or 0·8 mg. ascorbic acid daily and three groups received tablets of lucerne leaf-meal. The ascorbic acid was dissolved in 0·06 p.c. acetic acid and administered by a pipette. There were four female and three male animals in a group, the guinea-pigs being introduced into each in turn as they reached the required weight. When available, litter mates were distributed between the lucerne and corres-

[1] The experiments reported here were carried out before it was realized that it is inadvisable to include dried milk in basal diets for the bioassay of vitamin C.

ponding control groups. Four male animals received no supplement and served as negative controls. Animals of the same sex and group were housed together in large cages. During the period from 250 to 300 gm. weight they were removed to individual cages for part of each day till the ration of 10 gm. fresh lucerne was consumed. The experiment was continued for four weeks, when the guinea-pigs on the two upper levels of intake of vitamin were killed and their adrenals examined for vitamin C. Those ingesting 0·2 or 0·3 mg. ascorbic acid were continued in the experiment for 1 week longer.

Preparation and Analysis of Lucerne Tablets.

Fresh lucerne leaves were dried in a current of air at approximately 110° C. for 8 minutes. They were then finely ground and made up into tablets. These were placed in tubes which were evacuated and washed out with CO_2 twice and finally evacuated, sealed and stored in a refrigerator throughout the experiment. Each week one tube was opened and the week's supply of tablets for each guinea-pig weighed out. These were kept in a vessel which was evacuated and washed out with CO_2 and then evacuated again and kept in the refrigerator. It was opened every day to remove the day's tablets. It took fifteen weeks for the completion of the whole experiment, and the tablets from fourteen of the fifteen tubes were analysed at least once and frequently three times. Analyses for reduced and total ascorbic acid were carried out by Bessey's and by Lugg's methods. Analyses were several times made on the same batch of tablets on Monday and on Saturday and there was no falling off in either the reduced or total ascorbic acid in that time. Nor was there any appreciable change throughout the period of the experiment. The whole of the analyses may be summed up as follows:

Analysis of lucerne tablets. Ascorbic acid p.c.

	Lugg's method.		Bessey's method.	
	Reduced.	Total.	Reduced.	Total.
Means of all analyses	1·042	1·073	1·045	1·082
Tubes 2–7	1·044	1·076		
Tubes 8–13	1·040	1·071		

The lucerne leaf-meal was found to contain at most only a trace of reducing substances of the type of sulphite, cysteine, etc., which condense readily with formaldyhyde at pH 1·5 (Lugg, 1942). Reducers of the type which fail to condense with formaldehyde even at pH 3·5, such as ferrous salts, quinol and some reductones, were present in appreciable amounts, equivalent to about 4 p.c. of the total reducing capacity before treatment with H_2S and 6 p.c. afterwards. Mapson (1943) found that certain types of reductones condensed in part with formaldehyde and to that extent were estimated as ascorbic acid in Lugg's method. No evidence was found for the presence of "slowly condensing reductones" in lucerne extracts. On oxidizing such extracts with iodine and subsequently reducing by H_2S as recommended by Mapson, there was a decrease of 2 to 4 p.c. in the estimated total ascorbic acid as compared with the value found by Lugg's method. The reducing material in lucerne was stable on standing in metaphosphoric acid solution in air.

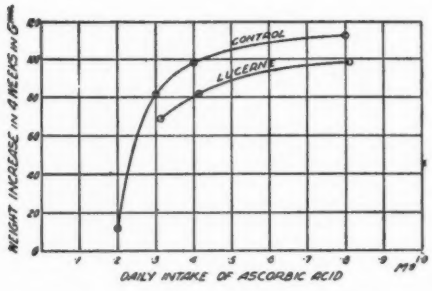

Fig. 1. Ordinate: Mean weight increments of groups of guinea-pigs in 4 weeks' experimental period.

Abscissa: Control, ascorbic acid intake. Lucerne, reduced ascorbic acid intake as measured chemically.

By whatever method it was estimated, the ascorbic acid in the lucerne tablets was greater than 1 p.c.

Mapson (1943) reported values for the ascorbic acid content of dried lucerne as determined by indophenol titration and by his modification of the formaldehyde method. After 6 minutes' drying at 100–105° C. the difference was 9 p.c.; after 15 minutes the value obtained by the formaldehyde method was only 64 p.c. of that found by simple titration.

Results of the Growth Experiment.

The mean weight increments of the guinea-pigs during the experimental period are presented in Table 1 and Fig. 1.

The negative control guinea-pigs died in 29–33 days.

TABLE 1.

Mean weight increments of guinea-pigs in growth experiment with lucerne leaf-meal (7 animals in each group).

Control.					Lucerne.						
	Weight increments.				A.A. daily intake.			Weight increments.			
A.A.	4 weeks.		5 weeks.					4 weeks.		5 weeks.	
daily		S.D. of		S.D. of	Reduced	Total A.A.			S.D. of		S.D. of
Intake.	Mean.	mean.	Mean.	mean.	A.A.	Lugg.	Bessey.	Mean.	mean.	Mean.	mean.
mg.	gm.	gm.	gm.	gm.	mg.	mg.	mg.	gm.	gm.	gm.	gm.
0·20	11·6	9·7	6·3	12·7							
0·30	81·6	7·0	96·9	11·1	0·31	0·32	0·32	68·7	8·1	71·9	10·7
0·40	98·9	8·4			0·415	0·43	0·43	82·1	15·9		
0·81	113·1	10·0			0·83	0·85	0·86	98·3	11·4		

It will be observed that at each level of intake of ascorbic acid the mean weight increment of the guinea-pigs ingesting lucerne was less than that of the animals receiving pure ascorbic acid. The differences between the means, and the standard deviations of the differences are shown in Table 2. It is apparent that the observed differences were not significant.

TABLE 2.

Differences between mean weight increments of control guinea-pigs and those ingesting lucerne.

Period.	A.A. intake.	Difference between means.	S.D. of differences.	Value of P.
weeks.	mg.	gm.	gm.	
4	0·3	12·9	11·6	0·3
4	0·4	16·8	19·4	0·4
4	0·8	14·8	16·3	0·4
5	0·3	25·0	15·4	0·1

ASCORBIC ACID IN THE TISSUES OF GUINEA PIGS.

At the conclusion of the lucerne growth experiment, the guinea-pigs receiving 0·4 mg. or 0·8 mg. of ascorbic acid or equivalent amounts of lucerne were killed with chloroform and the adrenals excised. They were extracted with metaphosphoric acid solution and the reduced ascorbic acid was determined by the method of Bessey (1938).

The ascorbic acid in the tissues of guinea-pigs has been measured by Fox and Levy (1936), Zilva (1936), Giroud, Leblond, Ratsimamanga and Gero (1938) and by other investigators. Kuether, Telford and Roe (1944) found that with a daily intake of 0·179 mg. ascorbic acid per 100 gm. body weight the adrenals of guinea-pigs contained 4·5 mg. p.c. With an intake of 1·23 mg. per 100 gm., which sufficed for normal growth and structure of all tissues, the amount of ascorbic acid in the adrenals was 53·2 mg. p.c.

The analyses carried out in the present experiment are summarized in Table 3. Despite the very small amounts present, there was distinctly more ascorbic acid in the adrenals of the guinea-pigs ingesting 0·8 mg. ascorbic acid than in those receiving 0·4 mg. The values for the lucerne and for the control animals did not differ significantly from one another.

The ascorbic acid in the liver of several of the animals was determined. The proportion of slowly reducing material was so great, however, and the amount of ascorbic acid so small, that the estimations of the latter were considered to be valueless.

TABLE 3.

Weight and ascorbic acid content of adrenal glands of guinea-pigs (7 animals in each group).

Control.				Lucerne.			
	Adrenals.				Adrenals.		
A.A.		A.A. content.		A.A.		A.A. content.	
daily			S.D. of	daily			S.D. of
intake.	Weight.	Mean.	mean.	intake.	Weight.	Mean.	mean.
mg.	gm.	mg.	mg.	mg.	gm.	mg.	mg.
0·4	0·25	0·013	0·003	0·4	0·23	0·016	0·003
0·8	0·22	0·023	0·001	0·8	0·24	0·021	0·002

DETERMINATION OF VITAMIN C BY HISTOLOGICAL EXAMINATION OF THE INCISOR TEETH OF GUINEA-PIGS.

The structural abnormalities which develop in the teeth of guinea-pigs in response to a dietary deficiency of vitamin C have been observed to vary with the degree of deprivation. A specific method for the measurement of vitamin C, which is based on these findings, has been elaborated by Höjer (1926), Goettsch (1928) and Key and Elphick (1931). This method was used to estimate the vitamin C content of dried lucerne and the results were compared with those obtained by chemical analysis.

Experimental.

Guinea-pigs weighing 208–300 gm. were housed in individual cages and fed on the basal diet of Coward and Kassner (1936) with halibut liver oil concentrate given separately. The dried milk was heated for 2 hours at approximately 105–110° C.

TABLE 4.

Influence of the vitamin C intake on the scurvy score of the incisor teeth of guinea-pigs.

Control.				Lucerne.						
					A.A. daily intake.					
					Reduced.		Total.			
No. of animals.	A.A. daily intake. mg.	Scurvy score mean.	S.D. of mean.	No. of animals.	Lugg. mg.	Bessey. mg.	Lugg. mg.	Bessey. mg.	Scurvy score mean.	S.D. of mean.
3	none	1·1	0·35							
5	0·19	1·52	0·32							
5	0·38	2·04	0·40	6	0·36	0·38	0·40	0·44	1·95	0·33
5	0·77	2·99	0·22	6	0·70	0·74	0·78	0·85	2·64	0·20

After one day without vitamin C, antiscorbutic supplements were administered for 14 days. Three groups of animals received ascorbic acid and two groups lucerne tablets. These were not prepared from the same sample of lucerne as those used in the growth experiment. They were stored in a refrigerator in air. One group of guinea-pigs without vitamin C served as a negative control. At the end of the experiment all the guinea-pigs were killed and the lower jaws excised. Transverse sections of the lower incisors were examined microscopically and scored by a method which was essentially that of Key and Elphick (1931).

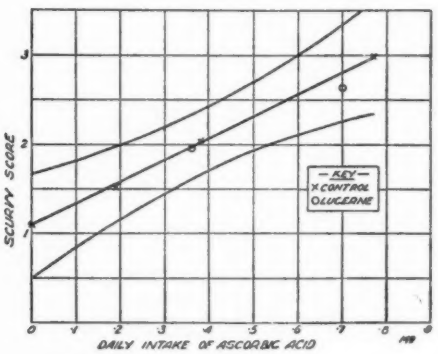

Fig. 2. Regression line of scurvy score on ascorbic acid intake of control guinea-pigs. Upper and lower curves represent fiducial limits of $P = 0{\cdot}05$.

Circles represent mean scurvy score of groups of guinea-pigs ingesting lucerne plotted against the reduced ascorbic acid intake measured chemically.

Results.

The results of this experiment are expressed in Table 4 and in Fig. 2. The regression function of scurvy score on ascorbic acid intake was a straight line, the equation for which was $y = 1{\cdot}08 + 2{\cdot}49x$ where y = scurvy score and x = ascorbic acid intake. From this graph the vitamin C intake of the animals receiving lucerne tablets could be deduced from their scurvy score. At the lower level of intake the vitamin C in the dried lucerne, as determined from the scurvy score, was approximately equal to the reduced ascorbic acid measured chemically. At the upper level it was about 10 p.c. less. However, it will be obvious from a glance at Fig. 2 that any difference between the vitamin C as measured chemically and by the tooth method was too small to be significant.

SUMMARY.

The vitamin C in dried lucerne was measured by its effect on the growth, and on the structure of the incisor teeth of guinea-pigs.

Its concentration as estimated by each of these methods appeared to be slightly less than that determined by chemical means.

However, the differences observed between the chemical and biological measurements of the vitamin C in lucerne were so small in relation to the variability of the animals as to be without statistical significance.

The ascorbic acid in the adrenal glands of guinea-pigs receiving lucerne was approximately equal in amount to that present in the adrenals of control animals receiving an equivalent amount of ascorbic acid as such.

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THE EFFECT OF INTAKE OF CAROTENE ON THE GENERAL HEALTH AND ON THE CONCENTRATION OF CAROTENE AND OF VITAMIN A IN THE BLOOD AND LIVER OF SHEEP

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Existing evidence suggests that vitamin A is the only known accessory food factor which is likely to limit the nutritional well-being of grazing sheep in Australia. When the pastures are green, the intake of the carotene-precursors of vitamin A by the grazing animal is greatly in excess of its immediate needs and only a small proportion of the quantity ingested is retained. That part of the vitamin which is assimilated and which is in excess of the current requirements is stored, principally in the liver.

The concentration of carotene in the pasture plants decreases as they mature, and by the time they are dry only a trace remains. Most Australian pastures are dry for several months each year, and in times of drought for much longer. Sheep then draw heavily on their reserves. The quantity of the vitamin A accumulated in their livers determines the length of the period that they can withstand dry feeding conditions without developing the disabilities of vitamin A deficiency.

There is considerable information available concerning the rôle of vitamin A in the nutrition of cattle, pigs, and poultry. Knowledge of the quantitative relationships of vitamin A in the case of sheep is, however, scanty. Guilbert, Miller and Hughes (1937) used evidence of night blindness as a criterion of an abnormal nutritional state in respect to vitamin A, and concluded that the sheep's minimum daily requirement was 25γ to 30γ of carotene or 6γ to 8γ of vitamin A per kg. of body weight. Schmidt (1941) considered that 14γ to 17γ of carotene per kg. live weight was sufficient to allow sheep to develop into "excellent physical specimens" although all the animals ingesting this amount were probably night blind. Underwood and Conochie (1941) and (1943) and Underwood and Curnow (1944) investigated various aspects of vitamin A in the nutrition of sheep in Western Australia. They described the fall in the sheep's liver reserves which supervenes during the dry period, and stressed the very low carotene concentration of the dry pasture species and of most of the supplements which are fed under these conditions.

The present paper reports the findings in the first of a series of investigations concerning the role of vitamin A in the nutrition of the Merino sheep which have been in progress in this laboratory for several years.

THE EXPERIMENT.

General Conduct.

The experiment was designed to study the effect of a low intake of carotene on the reserves of vitamin A in the liver and on the concentrations of vitamin A in the blood, and the overall effect on the general health of young sheep. A study was also made of the effects supervening on the addition of carotene to the ration when the reserves of vitamin A had been depleted.

Several criteria were used. The extent of storage was estimated in the livers collected from individuals which were slaughtered for the purpose. The animals were killed at intervals of approximately six weeks during the first nine months of the experiment and at somewhat longer

but irregular intervals, depending on the condition of the animal, during the remaining fourteen months. The concentrations of carotene and of vitamin A in the blood were estimated periodically. The animals were tested at intervals for the appearance of symptoms of night blindness, although from the beginning the difficulties associated with a reliable assessment of this manifestation of the deficiency were realized. Observations of the state of the retina were made on several occasions by means of the ophthalmoscope. Histological examination was made of the optic nerves and of the spinal cords of animals that were slaughtered when they had developed the terminal symptoms of the deficiency.

Experimental Animals and Treatment.

Sixteen wethers and two ewes, aged between four and eight months, which had been grazing on pasture and had been weaned approximately two months previously were selected at a period when the pasture was dry. The levels of carotene and of vitamin A in the blood of three of the wethers were estimated on three occasions during one week. These animals were then slaughtered and the concentrations of carotene and of vitamin A in their livers were determined. The remaining fifteen sheep were placed in individual pens and offered a diet consisting of 200 gm. chaffed wheaten hay, 250 gm. grain (wheat and barley in equal proportions) and 250 gm. linseed meal. The roughage was gradually replaced by chaffed wheaten straw and some molasses was added. By the tenth week the ration consisted of 200 gm. chaffed wheaten straw, 250 gm. grain, 250 gm. linseed meal and 100 gm. molasses. Four months later the ration was changed to 150 gm. of a compressed mixture of grain, straw chaff and molasses, 150 gm. straw chaff, 350 gm. barley and 300 gm. linseed meal. The carotene content of the various components of the ration was determined periodically. The limits of the ranges of the carotene contents are reported in Table 1.

TABLE 1.

Carotene content of components of rations.

	mg. carotene/kg.
Chaffed wheaten hay	5·2
Chaffed wheaten straw	0·20–0·43
Wheat and barley	0·31–0·48
Barley	0·33–0·89
Linseed meal	0·10–0·18
Compressed mixture	0·81

At the end of nine months by which time nine animals had been slaughtered and the remainder were known to be in a state of deficiency as indicated clinically by night blindness tests, by a low reserve of vitamin A in the liver and by a low concentration of vitamin A in the blood, these nine remaining animals were divided into three groups each of three animals. One group was continued on the basal ration; the individuals of the other two were given in addition sufficient dehydrated lucerne leaf meal to bring the daily intake of carotene to approximately 25γ and 50γ per kg. live weight, respectively. For this purpose, freshly dehydrated lucerne leaf meal was compressed into tablets and stored in this form in a refrigerator. The carotene content was determined at intervals, and from the chemical findings the amount of this material to supply the requisite amount of carotene for the ensuing week was calculated and withdrawn from the refrigerator. These tablets were kept at room temperature in a tightly closed tin from which the appropriate daily number of tablets was withdrawn and administered to each sheep by means of a "balling gun".

During the first nine weeks of the experiment while the fodder was being gradually changed to the basal diet, the mean daily intake of carotene fell from about 40γ to 20γ per kg. live weight. From then until the end of the nine months the intake was about 12γ per kg. For the remaining fourteen months of the experiment the animals on the basal diet received approximately 10γ, those on the intermediate level 25–30γ and those on the high level 50–55γ per kg.

Experimental Procedure.

The animals were weighed weekly.

Samples of blood from the jugular vein were collected into oxalate at intervals of approximately four weeks; bleeding was always carried out between 8 a.m. and 10 a.m. The blood was centrifuged immediately and the concentrations of carotene and of vitamin A in the plasma determined by a method similar to that of Kimble (1939). Ten ml. of plasma were pipetted into a 50 ml. centrifuge tube fitted with a glass stopper. To this, 10 ml. alcohol were added slowly, with continuous swirling of the tube. Finally, 15 ml. petroleum ether, b.p. 40–50° C. were added, the stopper sealed in with a drop of glycerine and the tube shaken vigorously by hand for about one minute. At intervals of not less than five minutes it was shaken for two further periods each of about one minute; between shakings the tube was kept in a beaker of

cold water to produce a slight negative pressure internally and thus prevent any loss of the volatile solvent. The tube was centrifuged at about 1,500 r.p.m. for five minutes and an aliquot of 10 ml. of the petroleum ether extract was pipetted into the photometer tube; care was necessary to prevent contamination with the alcoholic layer. The carotene was estimated by measuring the transmittance at 450 mμ. The tube was then placed in a water bath maintained at 40–50° C. and the petroleum ether removed in a stream of dry CO_2; a filter pump was coupled to the front of the system as it was found, in preliminary tests, that petroleum ether tended to condense in various parts of the system unless this precaution was taken. When evaporation of the petroleum ether was completed the outside of the tube was washed with distilled water and dried with a cloth, and the residue in the tube taken up in 1 ml. dry chloroform. To the contents of the photometer tube, 9 ml. Carr Price reagent were added from a fast delivery pipette. The intensity of the ensuing blue colour was determined by measuring, within 15 seconds, the transmittance at 620 mμ. For the estimation of the carotene and the vitamin A content of the livers of the animals which were slaughtered, the method of Underwood and Conochie (1941) was used. The content of carotene in the foodstuffs and in the lucerne meal was determined by the method of Peterson (1941). A Coleman Regional Spectrophotometer was employed and the transmittance calibrated, at 450 and 620 mμ, with standards prepared from S.M.A. carotene and S.M.A. crystalline vitamin A, respectively.

The petroleum ether fractions used in the determination of carotene in blood and in foodstuffs were those portions of Shell Solvent X.4 which boiled between 40 and 50° C. and between 50 and 70° C, respectively. These fractions were not purified further. The ethyl ether was purified by shaking anaesthetic ether with 10 p.c. of its volume of 10 p.c. KOH and allowing to stand overnight; it was then washed with water, dried over $CaCl_2$ and distilled. The chloroform was washed with water, dried over $CaCl_2$ and distilled. The ethyl alcohol used in the saponification of foodstuffs and of lucerne meal and the methyl alcohol used in the phase separation of carotenoid pigments were both purified by refluxing with KOH and distilling. The purified ethyl ether, chloroform, ethyl alcohol and methyl alcohol were stored in the refrigerator. The Carr Price reagent was prepared by dissolving A.R. antimony trichloride in freshly purified dry chloroform to a concentration of about 22·5 p.c. It was then filtered into 150 ml. pyrex flasks coated with black enamel and hermetically sealed. This reagent was stored at room temperature in the dark. A fresh flask was used for each set of determinations and the portion remaining was discarded.

The term "carotene" as used throughout this paper refers to the mixture of carotenoid pigments frequently called "crude carotene". It consists mainly of β–carotene with some α–carotene together with small amounts of other related pigments which are not removed in the conventional phase separation with 90 p.c. methyl alcohol.

The tests for night blindness were carried out at dusk, in a manner similar to that described by Guilbert *et al.* (1937). However, difficulty was found in assessing the ability of the animals to see in dim light. Some border-line cases were observed which were reported to be night blind on one occasion and to have normal vision on the next. Schmidt (1941) reported on similar difficulty in testing for night blindness in sheep. There is thus some uncertainty as to the reliance which can be placed on this determination as an early criterion of the state of deficiency in sheep. However, there was no doubt in assessing this symptom during the later stages.

RESULTS.

Effect on the General Health of the Animal.

In general, the animals remained in good health and ate a satisfactory amount of their ration until they were in an advanced stage of deficiency. All sheep killed in the first six months of the experiment were apparently in good health. One animal of the three that were receiving 25γ carotene per kg. body weight and two of those on the 50γ level were in good health when they were killed between the 22nd and 24th months of the experiment. One of the three on the highest level contracted balanitis in the 95th week, developed a stricture and had to be killed a few days later. There is, however, no reason to believe that a deficiency of vitamin A contributed to the cause of this disease. No calculi could be found in the kidneys or bladder.

The animals on the basal diet and the remaining two on the diet supplying 25γ per kg. remained in good health until a few weeks before death. Eye lesions were observed in only one individual. The animal had been receiving 25γ carotene per kg. live weight for five months after the period of depletion. A mucous discharge from the nose and mouth was observed. One eye lachrimated profusely and there were signs of conjunctivitis. The cornea at first slightly clouded became progressively worse until after a fortnight it was covered completely with an opaque film. This xerophthalmia, however, gradually improved, and, at the time of the animal's death, vision apparently was completely restored.

The most noticeable feature in the animals kept for a prolonged period on the intermediate and low levels of carotene was the muscular inco-ordination which appeared some weeks before death. All three animals on the low level and two of the three animals on the intermediate level

developed these terminal symptoms which first became apparent during the 51st week of the experiment. The affected animals when disturbed were unwilling to rise from the normal lying position—presumably because they found it difficult. When they attempted to walk, their hindquarters swayed and they were incapable of sustained effort. Histological examinations were made of the brain and spinal cords of three of them. In two cases a moderate degree of degeneration throughout the entire cord was observed, mainly in the ventro-medial tracts, the degeneration being most pronounced in the thoracic region.

From the 21st week the animals were tested at intervals for night blindness. Bearing in mind the lack of precision of this determination, the following observations are presented. At the 21st week only one animal was definitely night blind. Seven weeks later four additional animals were night blind. Six weeks later seven of the ten remaining sheep were night blind but it was uncertain whether the three remaining sheep were affected. Three weeks later, just prior to the time when the carotene was added to the ration of some of the animals, only one was not night blind. This animal which was confined to the deficient diet was definitely night blind when next tested seven weeks later, whereas by this time one animal on the 50γ level was no longer night blind and the vision of the other two of this group appeared to be improving. All the sheep on the deficient diet and two on the 25γ level continued to be night blind for the remainder of their lives. The three sheep on the 50γ level and one on the 25γ level ultimately recovered their ability to see in dim light. This last animal was the one which previously was mentioned as apparently being in good health at the time it was killed. Examinations by means of the ophthalmoscope revealed little abnormality, even in animals in an advanced state of deficiency. It would seem, therefore, that the papilloedema which Moore (1939) has observed in calves suffering from vitamin A deficiency is not a regular feature under similar circumstances in young sheep.

Effect on Body Weight.

The body weight increased steadily and satisfactorily in all animals for about 20 months (Fig. 1), but the weight of the animals in an advanced state of vitamin deficiency declined during the last few weeks of their lives. In some cases the decline in the consumption of food and the loss of weight were gradual, as the degree of deficiency advanced, but in others they occurred with noticeable suddenness.

Levels of Vitamin A and of Carotene in the Liver.

The values for the vitamin A concentration in the liver (Fig. 1) were obtained from single animals with the exception of the first which is the mean for three animals. The mean value for vitamin A in the liver of the three animals killed at the beginning of the experiment was 200γ vitamin A per gm. of liver; the individual concentrations being 120, 310, and 170γ vitamin A per gm. and their ages 17, 30 and 32 weeks, respectively. These lambs were born during the winter and were killed about two months after the grass had dried off. Their carotene intake during the previous two months had been low and so the concentration of vitamin A found in their livers was thus probably less than the maximum for young sheep grazing under natural

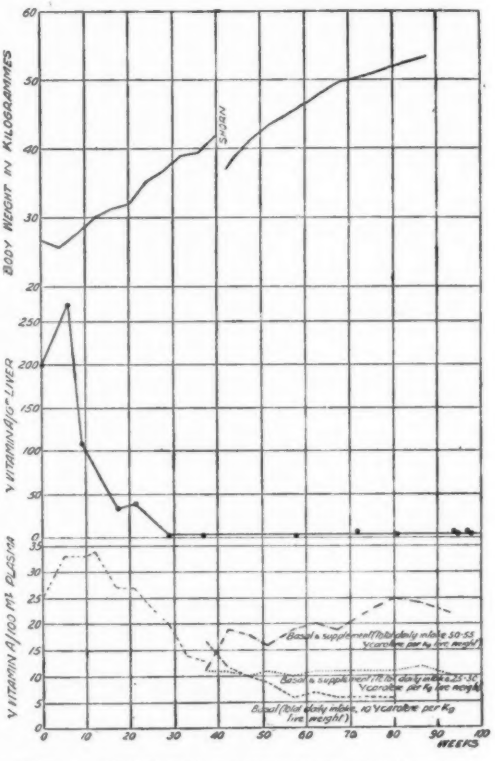
BODY WEIGHT IN KILOGRAMMES
SUSAN
VITAMIN A/g OF LIVER
VITAMIN A/100 ML PLASMA
Basal + supplement (Total daily intake 50-55 γ carotene per kg live weight)
Basal + supplement (Total daily intake 23-30 γ carotene per kg live weight)
Basal (Total daily intake, 10 γ carotene per kg live weight)
WEEKS

Fig. 1. Individual concentrations of vitamin A in the liver, mean monthly concentrations of vitamin A in the blood and mean monthly live weights of sheep.

conditions. However, these values were not particularly low as the concentrations of vitamin A in the livers of lambs four months old at the end of September in a subsequent year were found to be only 15 p.c. higher than the values mentioned above. The concentration in the animal which was slaughtered after six weeks of the experimental treatment, during which time its daily carotene intake had been approximately 45γ per kg. body weight, was 270γ vitamin A per gm. liver. From then on the values fell rapidly, to 35γ per gm., or 15 p.c. of the initial level by the end of the fourth month, and to an almost constant level of about 4γ per gm. by the end of the seventh month.

The amount of carotene found in the liver was always low. At the beginning, the livers contained between 1·5 and 2·5γ carotene per gm., i.e, about 0·5 p.c. of the total vitamin A potency. The findings indicated that the concentration of carotene in the livers fell slowly during the first four months, and then remained relatively constant at apparently 0·5γ to 1γ per gm. As the vitamin A concentration had fallen to a greater extent the proportion of the total vitamin A potency of the liver contributed by carotene increased; in the last 17 months of the experiment this was between 5 and 10 p.c.

The addition of sufficient carotene to the diet to increase the intakes either to 25γ or to 50γ per kg. body weight for five to fifteen months did not result in appreciable storage of vitamin A in the liver. The livers of the last four animals on the deficient diet had a mean value of 4γ vitamin A per gm. while those of the animals receiving 25γ and 50γ carotene per kg. live weight had mean values of 3γ and 7γ vitamin A per gm. of liver, respectively.

Levels of Vitamin A and of Carotene in the Blood.

The mean concentrations of vitamin A in the blood of the animals throughout the experiment are plotted in Fig. 1. The individual values for the first nine months of the experiment are shown in Table 2, while the values for the period during which varying amounts of carotene were added to the diet are shown in Table 3. During the first five weeks the initial value of 25γ vitamin A per 100 ml. plasma increased to 33γ. Presumably the intake by the young animals during the last few weeks grazing on dry pasture prior to the experiment was less than the 35–40γ carotene contained in the rations. The blood vitamin A remained relatively constant for the next two months; thereafter it declined progressively. At the end of six months the concentration was 70 p.c. and at the end of nine months only 40 p.c. of that during the first three months of the experiment.

TABLE 2.

Concentration of vitamin A in the blood of sheep for the first nine months of a low intake of carotene.

(γ vitamin A per 100 ml. plasma).

Sheep No.	Initial.	5 wks.	9 wks.	12 wks.	17 wks.	21 wks.	25 wks.	29 wks.	33 wks.	37 wks.
W 113	32									
W 115	27									
U 176	21									
W 150	18	21								
W 123	22	28	29							
U 168	24	44	39	35	22					
W 157	19	35	37	36	29	26				
U 174	21	34	30	27	20	13	13	12		
W 114	37	50	47	48	35	30	25	18	8	10
W 125	29	35	33	35	28	30	23	19	12	8
W 103	32	29	34	36	32	32	31	26	24	22
W 158	29	30	37	38	33	36	35	31	24	22
U 169	21	29	32	28	16	21	15	13	7	10
U 177	25	37	34	34	31	31	23	21	16	11
U 178	18	27	27	24	22	22	21	18	12	11
U 166	23	22	30	36	25	26	20	16	12	11
U 172	24	32	23	32	26	28	22	21	12	11
U 173	23	37	36	35	32	30	22	20	11	11
Mean	25	33	33	34	27	27	23	20	14	13

At the end of nine months, when additional carotene was provided, the concentration of vitamin A in the blood of the animals which received 50γ carotene per kg. rose abruptly within five weeks from 11γ to 19γ and thereafter more gradually to 24γ per 100 ml. plasma. The vitamin A in the blood of the animals receiving 25γ of carotene per kg. remained constant at about 11γ

vitamin A per 100 ml. plasma. The vitamin A concentration in the blood of the animals on the basal diet which provided about 10γ carotene per kg. body weight daily fell over this period from 17γ to 6γ vitamin A per 100 ml. plasma.

TABLE 3.

Concentrations of vitamin A in the blood of sheep from nine months onward. One group received the basal ration containing approximately 10γ of carotene per kg. live weight while the other two groups received sufficient additional carotene to raise the intakes to 25–30γ and to 50–55γ carotene per kg. live weight, respectively.

(γ vitamin A per 100 ml. plasma).

Sheep No.	Carotene intake γ/kg. live wt.	37 wks.	42 wks.	47 wks.	51 wks.	57 wks.	62 wks.	67 wks.	71 wks.	75 wks.	80 wks.	86 wks.	93 wks.
W 125	10	8	7	8	7	4	—	—	—	—	—	—	—
W 103		22	14	12	10	7	7	6	6	—	—	—	—
W 158		22	15	11	9	6	7	6	6	6	6	—	—
Mean		17	12	10	9	6	7	6	6	—	—	—	—
U 169	25–30	10	10	9	9	8	—	—	—	—	—	—	—
U 177		11	12	11	12	11	12	10	12	13	12	12	11
U 178		11	12	11	12	11	11	11	11	9	11	12	9
Mean		11	11	10	11	10	11	11	11	11	11	12	10
U 166	50–55	11	18	19	17	19	19	19	20	24	26	26	25
U 172		11	25	21	20	25	26	25	27	29	30	30	27
U 173		11	15	13	11	14	15	12	15	17	18	16	15
Mean		11	19	18	16	19	20	19	21	23	25	24	22

Only traces of carotene were observed in the blood; the concentrations were usually less than 2γ per 100 ml. plasma. The method of analysis employed was incapable of estimating such small amounts of carotene with precision. As the proportion was small this did not complicate the estimation of the vitamin A potency of the blood.

The relevant data for one animal, W 103, on the basal diet have been plotted in Fig. 2. The values shown here for concentrations in the liver are those obtained on the sheep slaughtered at intervals and may be presumed to reflect the changes occurring in the liver of this particular animal; the final value plotted is that obtained on analysis of its own liver after death. The graph depicts not only the steady increase in live weight which is characteristic of this experiment but also the abrupt cessation of this increase, coinciding with a reduced consumption of food, and the decline in weight in the last week of life.

FIG 2.

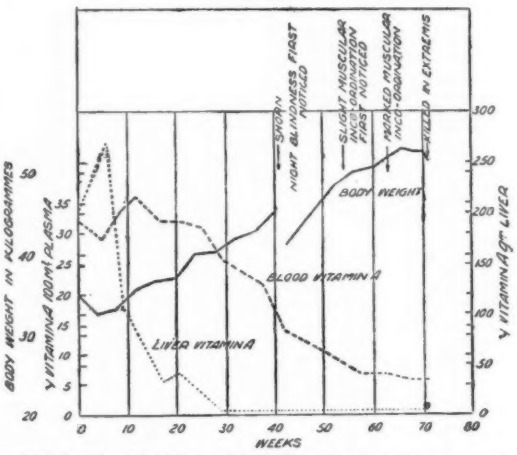

Fig. 2. Monthly live weights, monthly concentrations of vitamin A in the blood, together with the terminal concentration of vitamin A in the liver of W 103. The trend in the concentrations of vitamin A in the livers of sheep killed throughout the experiment is included for comparison.

DISCUSSION.

The diet which was deficient in carotene but adequate in other respects supported normal growth for about a year, after which period one animal developed muscular inco-ordination. Three more animals developed these symptoms dur-

ing the next two months and another after seventeen months had elapsed. Anorexia and consequent loss of weight supervened at this juncture. The observation that sheep will grow normally while subsisting on vitamin A deficient rations for approximately one year is in agreement with the findings of Schmidt (1941). Muscular inco-ordination has been observed in cattle and especially in pigs by many authors since the first report of this syndrome in pigs by Hart and McCollum (1914). The inco-ordination was later shown by Hughes, Lienhardt and Aubel (1929) to result from degeneration of nerves in portions of the spinal cord.

Clinical symptoms of a state of deficiency appeared much sooner. Night blindness was observed in one animal at five months. Thereafter the severity of the symptoms and the number of animals affected in this way increased. One animal, however, apparently retained its normal vision until some time between the eighth and tenth months. Guilbert, Miller and Hughes (1937) observed the first cases of night blindness in ewe lambs after they had been on similar rations for eight months; their remaining animals became affected within the next month. Schmidt (1941) observed that sheep one year old developed night blindness after five to six months on deficient rations. While the extent of the vitamin A reserves and the actual intake of carotene clearly determine the length of the period over which the animal can withstand the onset of these first specific clinical symptoms of vitamin A deficiency, individual variation of response also is involved. In our experiment the quantity of carotene suggested by Guilbert, Miller and Hughes (1937) as being sufficient to cure night blindness, namely 25–30γ carotene per kg. body weight, brought about recovery in only one of three animals. All the animals which received 50–55γ carotene per kg. per day recovered from night blindness, however, after from 7 to 57 weeks. Schmidt (1941) on the other hand, reported that levels of 55γ to 88γ carotene per kg. apparently did not restore vision in dim light to young sheep which had been receiving a ration deficient in carotene for upwards of one year.

The most striking consequence of the low intake of carotene was the rapid depletion of vitamin A in the liver. Within two months the concentration fell to 50 p.c., two months later to 15 p.c., and by the end of seven months to 2 p.c. of its initial value, at which level it remained until the last animal was slaughtered at 23 months. The corresponding mean values of 200, 100, 35 and 4γ vitamin A per gm. liver, respectively, are of the same order as those found by Underwood and Conochie (1941) who reported values of 600–1,000 I.U. vitamin A per gm. liver in mature sheep grazing on green pasture and of less than 50 I.U. in similar sheep after grazing for approximately six months on dry pasture. The figures obtained in the two experiments may be compared if it be assumed that 1γ vitamin A is equivalent to approximately 3 I.U. The small proportion of the vitamin A potency of the liver of sheep which is supplied by carotene is in conformity with the generally accepted belief that the sheep, in common with the goat and the pig, converts the greater portion of its ingested carotene into vitamin A. This is reflected in the white colour of the fat of these species due to the absence of carotenoid pigments. Palmer (1922) has commented on this feature of the body fats of these animals.

The concentration of vitamin A in the blood remained constant for the first three months. Subsequently it decreased slowly over the next three months to 70 p.c. of the initial concentration, and then, in the ensuing three months, more rapidly to 40 p.c. The minimum value, 20 p.c. of the original or 6γ vitamin A per 100 ml. plasma, was reached at about thirteen months and it

was maintained at this level until the end of the experiment. An intake of 25–30γ carotene per kg. body weight prevented this extreme fall and maintained the level at about 11γ per 100 ml. plasma. About double this intake, i.e., 50–55γ carotene per kg. body weight, increased the level to approximately 24γ vitamin A per 100 ml. plasma.

There was a loose correlation between level of vitamin A in the blood and the onset or disappearance of apparent night blindness. The concentration of vitamin A in the blood of the animal which was the last to develop night blindness was, for five months prior to the onset of the symptoms, consistently above that of the other animals which became affected earlier. The animal which first recovered after its intake of carotene had been raised to 50γ per kg. body weight also had a concentration of vitamin A in its blood which was consistently higher than that of the other animals on this level. With two exceptions, all the animals had low concentrations of vitamin A, 20γ per 100 ml. or less, when night blindness was first noticed. On the other hand, four out of the eleven animals showed symptoms of night blindness when the level of vitamin A in the blood had fallen below 25γ per 100 ml.; five were night blind when the level had first fallen below 20γ per 100 ml., and seven were night blind when the level had first fallen below 15γ per 100 ml. The level of vitamin A in the blood can thus be regarded as a more sensitive indication of a state of deficiency than the test for night blindness. This is not surprising since the clinical symptoms of nyctalopia do not lend themselves to accurate determination. The level of vitamin A in the liver of the sheep would, however, appear to be a more sensitive indication of the vitamin A status of the animal than is its level in the blood. The former falls rapidly when the intake of carotene is low, but, owing to the extent of the reserve, the level in the blood is maintained for some time. The level in the blood may still be within the normal range, which from this experiment appears to be 25–35γ vitamin A per 100 ml. plasma, when actually the concentration in the liver is very low. A low concentration in the blood, below 20γ per 100 ml., however, definitely indicates that a state of deficiency exists.

SUMMARY.

A diet which supplied daily approximately 10γ carotene per kg. body weight permitted satisfactory growth in young sheep for periods of upwards of one year. It resulted, however, in night blindness, low levels of vitamin A in the blood and liver, and eventually in anorexia, muscular inco-ordination and death.

Sheep four to eight months of age had an initial concentration of 200γ vitamin A per gm. liver. The concentration fell to 100γ at two months, to 35γ at four months and to 4γ per gm. after nine months. The concentration of vitamin A in the blood after remaining at the initial level, 35γ vitamin A per 100 ml. plasma, for three months fell proportionately more slowly; it reached 23γ at six months, 13γ at nine months and a constant level of 6γ per 100 ml. plasma from the thirteenth month.

The addition of a supplement of dried lucerne leaves to supply 25–30γ carotene per kg. body weight, which was provided to the animals after nine months on the deficient diet, arrested the fall in the level of vitamin A in the blood but had little beneficial effect on night blindness, and did not augment the remaining reserves of vitamin A in the liver.

A level of 50–55γ carotene per kg. body weight, given from nine months, brought about an increase of 100 p.c. in the level of vitamin A in the blood,

which was then raised to approximately 75 p.c. of the normal level; it cured the night blindness but had no appreciable effect on the extent of the reserves in the liver.

Acknowledgments. My thanks are due to Mr. I. W. McDonald and Dr. O. E. Nichterlein for the histological examinations of the nervous tissues, to Mr. F. Sergeant, of Thorpes Ltd. for the supply of the compressed feed mixture, to the Australian Wool Board for the financial support which made the experiment possible, and finally, to Mr. H. R. Marston, Chief of the Division of Biochemistry and General Nutrition, for his advice during the experiment and for his helpful criticism during the preparation and correction of the manuscript.

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STUDIES IN THE METABOLISM OF PLANT CELLS

4. THE REVERSIBILITY OF THE SALT RESPIRATION.

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(Accepted for publication 28th June, 1945.)

The previous papers in this series have shown that potassium chloride will raise the respiration—both oxygen consumption and carbon dioxide production—of cut carrot tissue and, further, that the mechanism of electrolyte accumulation is associated with this extra component of the respiration known as salt respiration. In two papers (1941, 1944) it was emphasized that the salt respiration once started remains constant at a given temperature, despite decreasing external and increasing internal concentrations of the salt. The purpose of this paper is to describe and discuss experiments which show that this salt respiration is a reversible effect and that complete removal of salt from the external solution results in marked reduction in the salt respiration.

MATERIAL AND METHODS.

Carrot tissue cut into discs was prepared in the same way as previously described (Robertson, 1941). Each set of tissue consisted of 80 discs, 1·5 cm. in diameter and 0·1 cm. thick. The carrots used in the first experiment were obtained from a different source from those used in the second experiment.

The tissue was kept in the apparatus in 240 ml. of distilled water or solution at 25° C. and aerated with carbon-dioxide-free air. In one experiment, the salt was applied by the addition of a small volume of a concentrated solution to the water; in the other experiment, the sets of tissue to be treated with salt were removed from aerated distilled water and placed in 240 ml. of salt solution. When desired the salt solution was subsequently replaced by drawing it off, rinsing the tissue and its container with distilled water and then adding a fresh 240 ml. of water. Salt uptake and carbon dioxide output were measured by the conductivity method described previously (Robertson, 1941). Both are expressed in gm. mol./set of tissue.

RESULTS.

Experiment I. (Figs. 1 and 2). Four replicate sets of tissue were used. After the initial period of equilibration in water, sufficient concentrated solution of potassium chloride was added to three of the replicates to give an external solution of 0·015 M. The potassium chloride caused an immediate increase in the rate of carbon dioxide production and initiated the process of accumulation. The salt respiration, i.e. the amount of carbon dioxide evolved by the treated tissue in excess of that evolved by the control in distilled water is shown in Fig. 1. The salt respiration rate remained steady so long as the salt was in contact with the tissue.

After the replicates had been in the solution for 40 hours, during which time the amounts accumulated were $9{\cdot}66 \times 10^{-4}$, $9{\cdot}50 \times 10^{-4}$ and $8{\cdot}85 \times 10^{-4}$ gm. mol respectively (Fig. 2), the salt was removed from replicates A and B which were rinsed once and then placed in distilled water. The conductivity of the distilled water surrounding the replicate A rose rapidly from that of the water (about 1×10^{-6} mho.) to about 3×10^{-6} mho. during the first 8 hours after the change. Subsequently it fell during the next 48 hours and reached an approximately steady value at $2{\cdot}1 \times 10^{-6}$ mho. If it were assumed that the whole increase in external conductivity (even at its maximum) were due to potassium chloride, i.e. that 2×10^{-6} mho. represented potassium chloride leached from the tissue, the total loss, $3{\cdot}4 \times 10^{-6}$ gm. mol., would be small compared with the amount accumulated, $9{\cdot}66 \times 10^{-4}$ gm. mol., i.e. only 0·35 p.c. Following the transfer back to distilled water, the respiration rate of replicates A and B gradually decreased almost to that of the control left in distilled water. The salt respiration of both A and B had been proceeding at $1{\cdot}55 \times 10^{-6}$ gm. mol./hr. but that of A decreased to $0{\cdot}20 \times 10^{-6}$ gm. mol./hr. and that of B decreased to $0{\cdot}40 \times 10^{-6}$ gm. mol./hr. The changes in rate (taken from the curve of best fit in Fig. 1) are shown in Fig. 3. After a further period, during which the total amount of potassium chloride accumulated by replicate C reached $12{\cdot}25 \times 10^{-4}$ gm. mol.,

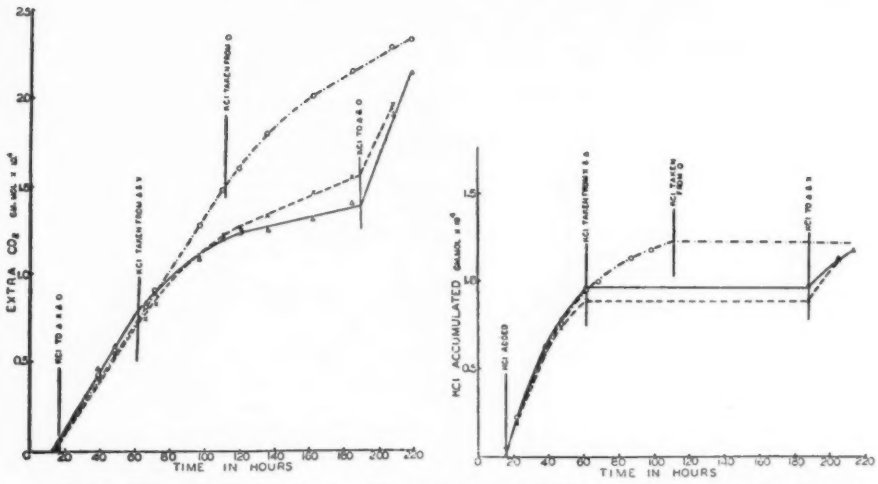

Fig. 1 and 2. Amounts of extra CO_2 evolved and of KCl accumulated, plotted against time, by tissue placed initially in a solution of 0·015 M KCl. Diacritic symbols: triangles for replicate A, crosses for replicate B, circles for replicate C.

this replicate was also transferred to distilled water. The same slow decline in respiration rate after the removal of the salt was observed. Finally, after the respiration rates of replicates A and B had fallen to the steady value, slightly above that of the control in water they were returned to salt and an increase in respiration rate took place. Both sets of tissue began to accumulate the salt immediately.

Experiment II (Figs. 4 and 5). This shows results similar to those of Experiment I, but with tissues initially in 0·01 M potassium chloride solution. The rate of the salt respiration in the replicate (C) in salt solution all the time showed a slight fall towards the end of the experiment; this fall was associated with the low concentration of salt left in the external solution by that time. After accumulation had proceeded for 54 hours, replicates A and B were transferred to distilled water. The same slow decline in the respiration rate as was observed in the first experiment took place. The salt respiration rate fell from $1{\cdot}86 \times 10^{-6}$ gm. mol./hr. to $0{\cdot}42 \times 10^{-6}$ gm. mol./hr in set A and from $1{\cdot}92 \times 10^{-6}$ gm. mol./hr. to $0{\cdot}38 \times 10^{-6}$ gm. mol./hr. in set B. The loss of electrolyte on transfer of the rinsed tissue to distilled water was again negligible compared with the electrolyte previously accumulated.

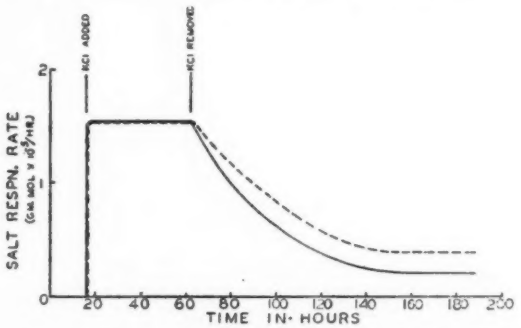

Fig. 3. Rate of salt respiration (rate of extra CO_2 production) by two replicates (A, continuous line, and B, broken line).

Experiment III. To ascertain whether accumulated salt was lost rapidly to the rinsing water another experiment was carried out. A set of tissue slices, 40 discs, each 1 mm. thick and 8 mm. in diameter, was placed in 34·5 ml.[1] of aerated 0·01 M potassium chloride solution. After 22 hours, $0{\cdot}56 \times 10^{-4}$ gm. mol. potassium chloride had been accumulated. The tissue was then quickly rinsed with three successive changes each of 34·5 ml. distilled water and the increases in the conductivity of the water after rinsing were respectively $18{\cdot}3 \times 10^{-6}$, $0{\cdot}7 \times 10^{-6}$ and

[1] The ratio of tissue volume to solution volume is that of the earlier experiments.

$0{\cdot}1 \times 10^{-6}$ mho. A similar set of tissue was placed in 0·01 M potassium chloride solution for 15 min.; this is long enough to allow the salt to diffuse through the tissue but not long enough to allow any appreciable accumulation. The tissue was then rinsed with three successive changes each of 34·5 ml. distilled water and the increases in conductivity were respectively $17{\cdot}5 \times 10^{-6}$, $5{\cdot}5 \times 10^{-6}$ and $1{\cdot}9 \times 10^{-6}$ mho. From the similarity of the increase in conductivity in rinsing these two sets of tissue, it may be concluded that the water used in rinsing removes the salt from the surface of the discs, from intercellular spaces and from cell walls but does not have an appreciable effect on salt accumulated.

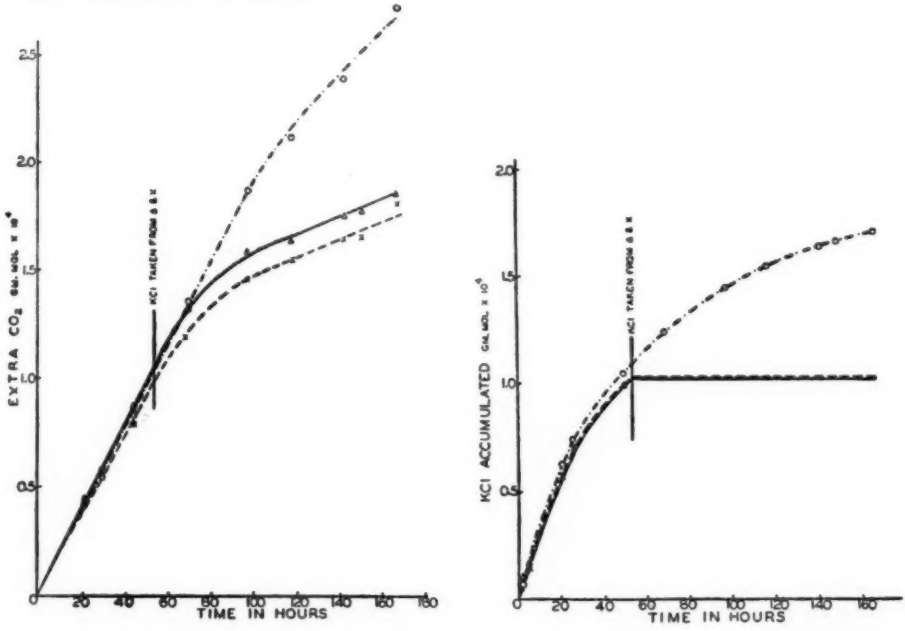

Fig. 4 and 5. Amounts of extra CO_2 evolved and of KCl accumulated, plotted against time, by tissue placed initially in a solution of 0·01 M KCl. Diacritic symbols: triangles for replicate A, crosses for replicate B, circles for replicate C.

DISCUSSION.

The Absorbed Salt.

The results presented in this paper show that the accumulatory mechanism operates to raise the salt to a level of high concentration in the cells and that this salt is not lost when that in the external solution is removed. The total lost —even if it were all potassium chloride—is very small and, as has been shown by Briggs and Robertson (unpublished data), the rise in conductivity of water surrounding tissue can be accounted for largely by the bicarbonates formed in the external solution by equilibration of cations from the tissue with carbon dioxide from respiration. This maintenance of the concentration accumulated, despite change of the external solution, is then, an important attribute of the cell.

The Reversible Salt Respiration.

It can be seen from the results presented here, as well as those given in earlier papers, that the salt respiration remains steady over a long period of time provided that the salt is maintained in the external solution. It has been shown

(Robertson and Turner, 1945) that the salt respiration is independent of external concentration at least over the higher concentrations and this independence continues as the salt concentration inside the cell rises and as that in the external solution falls.

When, however, the tissue is transferred to water and the external salt completely removed, the salt respiration declines. The slow decline (half time about 20 hours) in the rate of of salt respiration which is shown in Fig. 3 is in striking contrast to the rapidity with which the process is initiated (half time about 20 minutes), (Robertson and Turner, 1945). After the rate has declined till the salt respiration is only about 30 p.c. of its maximum, fresh application of salt to the external solution results in a rapid rise in the salt respiration.

The Nature of the Process and its Relation to Cell Organization.

We have established that tissue which has accumulated electrolyte shows practically no loss on return to distilled water, and we can now state that :

(a) high salt respiration is not responsible for the *maintenance* of the high internal salt content; for when salt respiration is inhibited by cyanide, no loss of electrolyte occurs (Robertson and Turner, 1945) and,

(b) high internal salt concentration is not responsible for the maintenance of high salt respiration; for when tissue is returned to water, the respiration returns almost to that of the control while the internal salt concentration remains high.

We may conclude therefore that the salt respiration is closely associated with the process of accumulation and that the force which retains ions in the cell is quite different from that which accumulates the ions in the region of high concentration. It is signficant that the salt respiration does not fall to zero after the removal of salt from the external solution. It is possible that this residual salt respiration (not exceeding 30 p.c. of the maximum) represents an effect of the high concentration of ions accumulated.

In Parts I and III of this series, it was shown that the rate of salt respiration is limited by the external salt concentration when this is low (*circa* 0·0044 M) and that the rate of salt respiration is independent of external concentration when this is high. We might therefore expect a fall in the rate of the salt respiration when the external salt solution is replaced by distilled water (which becomes a very dilute solution). This fall is obtained, but the decline to the low rate takes about 60 times as long as the original rise (Fig. 3). To explain this slow decline there are at least three possibilities:

(1) Removal of salt from the region where it affects respiration may be slow. It has been shown that practically no salt is lost to the external solution but possibly the salt which affects respiration is moved slowly to the region of accumulation. The results of S. C. Brooks (1940) suggest that radioactive substances pass only slowly from the cytoplasm to the cell sap.

(2) Reversion of enzyme systems to the original state might be slow even if an (hypothetical) enzyme modification brought about by the electrolyte were rapid. Ions might for instance sufficiently affect an interfacial layer at which a protein is spread to alter radically the configuration of the protein and hence initiate enzyme action. Replacement of ions allowing a reversion of structure might then take a measurable time.

(3) Complete utilization of a new substrate liberated by the action of salt might be slow. If the salt liberated a new substrate, either directly or by causing an enzyme change, then on removal of the salt, the respiration would revert to normal only when the substrate had been exhausted.

Relationship to Other Work.

These observations are related to the controversy on salt respiration. Lundegårdh and Burström showed, as long ago as 1933, that salt raised the respiration of cells which had been in water; this rise was associated more closely with the amount of anion than with the amount of cation entering. As pointed out previously (Robertson, 1941), the amount of cation is usually in great excess of the amount of anion entering because a large number of cations are exchanged, whereas the anions are practically all accumulated. The amount of an anion is therefore representative of the amount accumulated, and it is accumulation (not exchange) and salt respiration which are related. Steward, Stout and Preston (1940) and Hoagland and Steward (1940) recognized that salt does affect the respiration but possibly techniques employed by Hoagland and his collaborators did not allow the magnitude of the salt respiration to be appreciated. The usual technique in accumulation experiments (Hoagland and Broyer, 1936) is to remove material from culture solution and to place it, after rinsing, in a solution of electrolyte. Salt respiration, if induced by the culture solution, is likely to be still present and the effect of the electrolyte in the experiment would, in consequence, appear slight. A persistent effect from the culture solution might explain the very slight difference between the carbon dioxide production of barley roots in distilled water and that of roots in salt solution during the short experimental period of 5 to 8 hours (Hoagland and Broyer, 1942) and might also influence the results of Steward, Stout and Preston (1940). There has, however, been no investigation of whether a balanced solution (or one containing ions showing antagonism) stimulates a salt respiration.

Lundegårdh working with wheat roots (c.f. Lundegårdh, 1940) found that the distilled water respiration rate exceeded that of the ground respiration, obtained from extrapolation of the curve for respiration rate plotted against amount of anion absorbed. This may have been due to the salt respiration stimulated by the culture solution persisting during the experimental period.

SUMMARY.

This paper shows that:

(i) the salt respiration in carrot tissue is reversible;
(ii) the fall in respiration rate on removal of salt takes about 60 times as long as the rise on application of the salt;
(iii) the fall in salt respiration rate is not associated with loss of electrolyte previously accumulated in high concentration.

The relationship of these results to other work is discussed.

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THE QUANTITATIVE EXTRACTION OF COBALT AND IRON FROM ASHED BIOLOGICAL MATERIAL

by A. B. BECK[1]

(From the Division of Animal Health and Production, Council for Scientific and Industrial Research).

(Accepted for publication 24th August, 1945.)

In the investigation of "trace element" deficiencies in plant or animal material it is an obvious advantage to be able to determine the concentrations of a number of elements on one sample; most existing methods allow for the determination of only one or perhaps two. The degree of separation of these elements by solution of organic complexes in immiscible solvents is influenced by pH, by the concentration of sulphate ions (from wet digestion) and of citrate ions (added to prevent precipitation in alkaline solutions), but complete data on the effects of these factors are not given in existing literature.

In 1943 an investigation was begun to obtain further information on these points and also to evolve simple, yet strictly quantitative separations of a number of elements. This investigation has had to be suspended for an indefinite period on account of more urgent work, but it is considered that the results obtained so far are of value, particularly where it is required to separate cobalt quantitatively from other elements.

The conditions for the quantitative extraction of iron by solution of the cupferron complex in chloroform, and the possibility of separating iron from copper and cobalt by this method, have been investigated.

The extraction of cobalt both as dithizone and 1-nitroso-2-naphthol complexes has been examined, and as a result of these investigations a scheme is suggested for the quantitative separation of copper, cobalt, zinc, lead and iron from solutions obtained after the ashing of biological material. The scheme has been used for the examination of samples and has given completely satisfactory results.

EXPERIMENTAL.

General.

A visual colorimeter was used for comparison with standard solutions in most of the estimations made. Tall Nessler tubes (19 cm.) were used for amounts of less than 1 microgram of cobalt, which were determined by a slight modification of the nitroso-R-salt method of Marston and Dewey (1940) and Bayliss and Pickering (1941). Small amounts of zinc and lead were estimated by the mixed-colour dithizone method similar to that used for lead by Clifford and Wichmann (1936). Larger amounts of zinc were estimated by the dithizone colorimetric method (Hibbard, 1937), copper as the diethyldithiocarbamate complex in amyl alcohol (Sylvester and Lampitt, 1935) and iron as the dipyridyl complex (Jackson, 1938).

For the determination of extraction curves, aqueous solutions containing the element as sulphate or chloride were used: 3 ml. H_2SO_4 (representing the maximum amount likely to be present after wet digestion) and varying amounts of citric acid (as ammonium citrate purified by dithizone) were added in each case. The approximate pH was obtained by the addition of ammonium hydroxide, using internal indicators, and the volume made to 100 ml. After extraction of the element, the exact pH was determined with the glass electrode. All extractions were done at 22–23° C. The quantities of reagents, times of shaking, etc., had to be decided arbitrarily, and where possible are on the same basis as those used by other workers.

In the colorimetric estimation of cobalt, the same amount of sulphuric acid was added to the standards as to the actual estimation to ensure comparable conditions. It was found that in all grades of sulphuric acid available (including glass distilled) there was a small amount of some

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material which caused decreased colour formation. Digestion of the sulphuric acid with a little perchloric and nitric acids completely removed the interfering substance.

Chloroform was purified according to the procedure of Bambach and Burkey (1942).

Extraction of Iron with Cupferron.

Estimation of iron with dipyridyl directly on aliquots from a wet digest sometimes gives low results (presumably due to phosphates). This can be overcome by separation of the iron as sulphide (Jackson, 1938) or by checking the colour intensity again after twenty-four hours (Koenig and Johnson, 1942). The second method is not considered desirable, while the first, although giving excellent results, is cumbersome and not selective. A simpler quantitative separation would be desirable not only for accurate estimation, but also because iron interferes in certain colour reactions, and may produce precipitates which absorb other elements. In these cases citrate is usually used to depress the ionization of iron, but complete removal would be preferable.

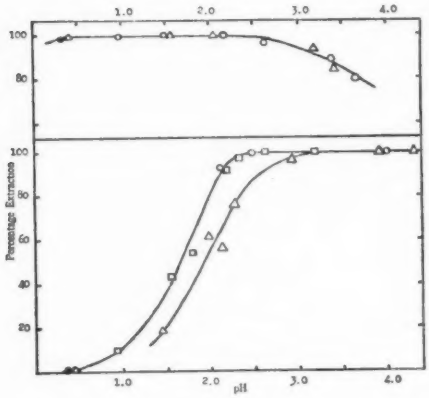

Fig. 1. Top curve. Extraction of iron as cupferron complex in $CHCl_3$.

—●——●— No citric acid.
—△——△— 0·75 p.c. citric acid.
—○——○— 4·0 p.c. citric acid.

Bottom curves. Extraction of copper as cupferron complex in $CHCl_3$.

—●——●— No citric acid. 200 p.c. excess cupferron.
—△——△— 0·75 p.c. citric acid. 50 p.c. excess cupferron.
—○——○— 0·75 p.c. citric acid. 200 p.c. excess cupferron.
—·□——□--- 4·0 p.c. citric acid. 200 p.c. excess cupferron.

Cronheim (1942), in the estimation of relatively large amounts of cobalt (above 20 micrograms), suggests that iron could be precipitated first from a strongly acid solution as the cupferron complex but he gives no details. This complex is readily soluble in chloroform, but as far as is known, no studies have been made to determine the possibilities of using this property for quantitative separation of iron from biological ashes, although the method is widely used in general analyses (Baudisch, 1909).

The extraction of iron was studied on a solution containing 5 mg. Fe^{+++}, 3 ml. H_2SO_4, and 0·75 or 4·0 gm. citric acid. Ammonia was added to the required pH and the volume made to 100 ml. After saturating with chloroform, 52 mg. cupferron (25 p.c. excess of theory) was added as 1 p.c. solution. The solutions were allowed to stand exactly 10 minutes, 5·00 ml. $CHCl_3$ added, and the separating funnels shaken vigorously by hand for exactly one minute. After standing, 1·00 ml. of the $CHCl_3$ layer was pipetted off and the Fe content determined after evaporation and digestion with H_2SO_4, HNO_3 and $HClO_4$. The results are shown in Fig. 1. Iron can be almost quantitatively extracted (99 p.c.) in the presence of citrate and sulphate using 25 p.c. excess cupferron at pH values between 1 and 2 (thymol blue indicator). Although it appears from Fig. 1 that quantitative recoveries are obtained, a small amount of iron is left in solution. This is readily removed by a second treatment with cupferron. Ether and carbon tetrachloride also dissolve the Fe-cupferron complex and presumably would give similar extraction curves. There is a definite time factor involved in the reaction of iron with cupferron at pH values above 3; longer standing gives increased complex formation.

Extraction of Copper and Cobalt with Cupferron.

The extraction of copper from a solution of $CuSO_4$ containing 2 mg. Cu per 100 ml. was studied under exactly the same conditions. With 25 p.c. in excess of the theoretical amount of cupferron, poor recoveries were obtained, so excesses of 50 p.c. and 200 p.c. were used. The results are shown in Fig. 1. It will be noted that in the most acid solution containing 3 ml. H_2SO_4 per 100 ml., 0·8 p.c. of the copper is still extracted. Therefore, the quantitative separation of iron from copper does not appear to be possible.

The extraction of cobalt by cupferron has not been studied completely. With 20γ Co in the presence of 3 ml. H_2SO_4, 0·75 gm. citric acid per 100 ml. and ammonia to the required pH, no cobalt is extracted using 50 mg. cupferron and chloroform at pH values below 2. At pH 3–4 small traces (0·1γ) are extracted. Using 200 mg. cupferron, a small amount of Co is extracted even at pH 1·4 and at pH 3–4 a large percentage of the cobalt passes into the chloroform. If, however a cupferron separation is done on solutions of pH 0·5 to 1·5 and containing 5 mg. Fe and 4γ Co (with citrate and sulphate), incomplete recoveries of cobalt (around 90 p.c.) are obtained; this indicates that co-precipitation and subsequent extraction occur even at low pH values.

These experiments indicate that cupferron is not satisfactory for separating iron in the presence of copper or cobalt. Iron however, may be quantitatively separated from the wet digest *after* the removal of dithizone-extractable metals, by reacidifying, forming the cupferron complex, and extracting with $CHCl_3$ or CCl_4. In this way iron may be separated from all materials which are likely to interfere with the determination by dipyridyl.

Extraction of Cobalt with Dithizone.

In the dithizone method of Marston and Dewey (1940), sulphate must be absent and the citrate concentration below 0·03 M. In the method of Parks *et al.* (1943) sulphate is absent and the citrate concentration is about 0·2 M, yet quantitative recoveries are obtained. Sylvester and Lampitt (1940) extract cobalt quantitatively in the presence of both sulphate and citrate. This difference in the effect of these two ions in the different methods has been confirmed. The only essential difference is that Marston and Dewey use sodium salts for neutralization and buffering, whereas the other workers use ammonia salts, and the difference in extractions must be due to this fact.

Although Marston and Dewey give complete pH-extraction curves for their technique, no curves seem to have been reported for the extraction using ammonium hydroxide and citrate for neutralization and buffering. Twenty γ of Co were taken in a solution containing 3 ml. sulphuric acid, 1·0 or 4·0 gm. citric acid, and ammonia to the required pH. The volume was made up to 100 ml. and the solution shaken by hand for 2 minutes with 5·00 ml. of 0·025 p.c. purified dithizone in CCl_4 which had been standardized by extractive titration against silver sulphate (Fischer, Leopoldi and Uslar, 1935). After standing, a suitable aliquot of the CCl_4 was pipetted off for analysis. The results are shown in Fig. 2. It is apparent that quantitative recoveries are possible in the presence of sulphate and 0·2 M citrate, provided ammonia is used for neutralization. Small amounts of cobalt are extracted even at pH 4, although the quantities are much smaller than those recorded by Marston and Dewey. It should be pointed out, however, that the curves shown in Fig. 2 represent extractions with two-minute shakings, whereas the above workers give values approximating to equilibrium. The position of points below maximum extraction on the acid side will be raised by more intense or more prolonged shaking. A few extractions have been done using 0·025 p.c. dithizone in chloroform and the results confirm the findings of Marston and Dewey that the extraction curve is shifted considerably to the right, and that no cobalt is extracted at pH 3 if chloroform is used as the solvent, even with more concentrated solution of dithizone.

Parks *et al.* (1943) separate zinc from cobalt by shaking the alkaline dithizone extract with 0·02 N hydrochloric acid. The cobalt remains in the CCl_4 phase, but as has been pointed out by Walkley (1942) the decomposition of zinc dithizone by dilute acid is slow and incomplete. The use of stronger acid (up to 0·1 N) gives better recoveries of zinc without removal of cobalt. Although not strictly quantitative, the separation is sufficiently complete for ordinary use. The nitroso-naphthol separation described in the next section is preferred however, as it is more specific.

Separation of Cobalt with 1–Nitroso–2–Naphthol.

Although it has been known for many years that the cobalt complex of 1–nitroso–2–naphthol is soluble in chloroform, this fact does not seem to have been used to separate cobalt from biological ashes, apparently because iron and copper form similar complexes. The following scheme was evolved for separating cobalt from the alkaline-dithizone extract, which may contain Zn, Pb, Co and probably Ni, Bi and Cd.

Digest the dithizone extract with H_2SO_4 and $HClO_4$, dilute, add 10 p.c. citric acid and ammonia to pH 6 (brom-cresol purple), warm to 60° C. or more, transfer to a separating funnel and add 0·2 ml. of 2·5 p.c. nitroso–naphthol in acetone. Allow to stand for thirty minutes or more, and extract the cobalt complex with chloroform. This method is slightly cumbersome, but the separation is specific for cobalt and the reagent concentrations and pH are not critical.

This procedure can also be used with success on biological ashes, provided copper is first removed at pH 3 by dithizone. In the presence of adequate citrate the reaction with iron is extremely slight. There is some evidence that incomplete oxidation of the organic matter (as

sometimes occurs with liver tissue) may cause low results, but no difficulty has been experienced with well digested samples.

At this stage of the investigation a paper by Alexander and Taylor (1944) was noted in which the writers carry out a preliminary separation of cobalt, prior to zinc determination, by shaking the solution (containing citrate) at pH 8·2–8·4 with 0·05 p.c. nitroso–naphthol in chloroform. This technique appeared to be more convenient than the method then in use, so the extraction curve was studied. A solution containing forty γ Co, 3 ml. H_2SO_4 and 4·0 gm. citric acid was brought to the required pH with ammonia and the volume made to 100 ml. After saturating with chloroform and removing excess, 10·0 ml. of freshly prepared 0·05 p.c. nitroso–naphthol in chloroform were added and the separating funnel vigorously shaken for two minutes After standing, a suitable aliquot of the $CHCl_3$ was pipetted off and the cobalt content determined. The results are shown in Fig. 3.

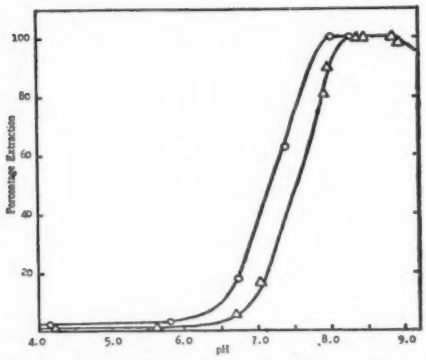

Fig. 2. Extraction of cobalt by dithizone in carbon tetrachloride.

—O——O— 1·0 p.c. citric acid.

—△——△— 4·0 p.c. citric acid.

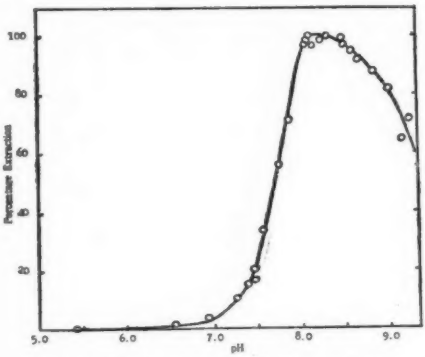

Fig. 3. Extraction of cobalt by 0·05 p.c. nitroso–naphthol in chloroform.

It will be noted that the extractions above about pH 8 tend to be erratic. The cobalt remaining after a single shaking reacts only partially when a second extraction is made. Apparently cobalt–ammonia complexes are formed which react very slowly with nitroso–naphthol at room temperatures. For maximum recoveries the pH must be close to 8·2 and it is practically impossible to obtain such accuracy with wet digest solutions using internal indicators. It is considered that although cobalt can be extracted by the method in its present form, the conditions for complete extraction are too critical for ordinary use.

It should be emphasized that these findings in no way invalidate the claim by Alexander and Taylor that their technique quantitatively separates cobalt from zinc. Any cobalt not extracted by nitroso–naphthol will remain in the CCl_4 phase when the zinc and cobalt dithizonates are decomposed by dilute acid.

Carbon tetrachloride gives a maximum extraction at a lower pH, but it is not satisfactory as the solubility of the cobalt complex in this solvent is extremely low.

SUGGESTED SCHEME FOR THE SEPARATION OF TRACE ELEMENTS.

A method for the estimation of 12 elements on one sample has been put forward by Parks *et al.* (1943), but their scheme for the trace elements has not been followed for two main reasons. As has already been pointed out, the separation of zinc and cobalt is not quantitative. These writers also do a number of their estimations with selective reagents in the presence of relatively large amounts of other elements. This technique is time-saving, and in some cases

cannot be avoided, but it may not always give reliable results, particularly on materials of unknown composition. In such cases it is highly desirable to separate the element to be estimated from the other elements and radicals present.

The following tentative scheme of separation makes no claim to cover all trace elements, but is adequate for most deficiency investigations. The separations are quantitative and the reagent concentrations and pH values are not critical. As far as the method has been tested it has given completely satisfactory results. All reagents must of course be "free" from the elements to be tested and "blanks" should be done with each batch.

Digest the sample (usually 10 gm.) with 3 ml. H_2SO_4, 2–3 ml. $HClO_4$ (70 p.c.) and nitric acid (about 70 ml. for 10 gm. samples). Add additional HNO_3 and $HClO_4$ until oxidation is complete. With liver samples the digest frequently takes on a brownish tint after the perchloric acid has been fumed off; it is usually necessary to drop a mixture of HNO_3 and $HClO_4$ (2: 1) on to the fuming sulphuric acid in order to oxidize completely the last traces of organic matter.

If the material is very high in iron (above about 10 mg. Fe) dilute with 8 N HCl and extract the bulk of the iron with isopropyl ether saturated with HCl. Boil off the HCl and take down to fumes. This stage is not necessary with ordinary materials.

Dilute with a little water and add a few drops of SO_2 solution to remove any material likely to oxidize dithizone. Boil to remove excess SO_2. Add a slight excess of bromine water, allow to stand a few minutes then boil off excess bromine. If the material is high in Ca, or if Pb is to be estimated, add 5 ml. 20 p.c. HCl to assist solution of the sulphates. To the warm solution add 10 p.c. citric acid (as ammonium citrate), sufficient to prevent precipitation at pH 8; for unknown material 40 ml. is used, but if this can be reduced to 20 or even 10 ml. more rapid extraction of cobalt and zinc will be obtained. Add a minimum amount of brom–phenol–blue indicator and partly neutralize with ammonia. Warm to ensure complete formation of ferric citrate, and filter through an acid-washed filter paper. Bring to pH 3 with ammonia (check externally with brom–phenol–blue). Cool if necessary and extract copper completely with 0·2 p.c. dithizone in chloroform. Evaporate the chloroform, digest the residue with H_2SO_4 and $HClO_4$ and estimate the copper as the dithiocarbamate complex.

If cobalt and iron only are to be extracted, brom–cresol–purple indicator is added to the solution which is brought to pH 6 (definite purple) with ammonia. If however, zinc and lead are to be extracted afterwards, this indicator will mask the phenolphthalein colour change at that stage; in this case the indicator must be used externally (between 6·5 and 8·5 ml. of 5 N ammonium hydroxide will be needed to bring the solution to pH 6). Warm to 60° C. or more, transfer back to the separating funnel, add 0·2 ml. of 2·5 p.c. nitroso–naphthol in acetone. Allow to stand 30 minutes or more and extract with three lots of 5 ml. $CHCl_3$ (one minute shakings). Combine extracts, evaporate, digest thoroughly with 0·5 ml. H_2SO_4 and a few drops of $HClO_4$ and HNO_3; estimate cobalt as the nitroso–R–salt complex. To the aqueous solution after the removal of cobalt, add ammonia to pH 8·2–8·4 (phenolphthalein). It is probable that nickel can be extracted quantitatively at this stage using dimethyl–glyoxime and chloroform (Alexander and Taylor, 1944) but this point was not investigated. Extract zinc and lead with dithizone in CCl_4; these elements may be estimated by standard procedures. Acidify slightly and wash out excess dithizone with $CHCl_3$ or CCl_4; add thymol blue indicator and HCl or H_2SO_4 to pH 1–2. Boil up with a little bromine water to ensure that all iron is in the ferric state. Cool thoroughly, add excess cupferron, allow to stand 10 minutes or more; extract with $CHCl_3$ or CCl_4. Add more cupferron to ensure complete complex formation, allow to stand and re-extract. Evaporate the solvent and digest the residue cautiously with 0·5 ml. H_2SO_4 and a few drops of $HClO_4$ and HNO_3. If a preliminary isopropyl ether extraction has been done, combine extracts and estimate the iron by dipyridyl or other standard method.

If cobalt only is to be estimated, dithizone is the more convenient reagent, provided there is no danger of precipitates forming at pH 8·4 and provided that the quantities of zinc or lead are not excessive. If these conditions exist, nitroso–naphthol should be used.

SUMMARY.

The extraction of copper, iron, and cobalt by solution of the cupferron complex in chloroform has been studied at pH values between 0·4 and 4. It was not found possible to separate quantitatively iron from copper and cobalt.

Data are given for the extraction of cobalt by dithizone in carbon tetrachloride and by 1-nitroso-2-naphthol in chloroform at various pH values.

A scheme for the separation of "trace elements", largely based on existing procedures, is given. Copper is extracted by dithizone in chloroform at pH 3, cobalt at pH 6 by nitroso-naphthol and chloroform, and zinc and lead at pH 8·4 with dithizone in carbon tetrachloride. After reacidifying to pH 1–2, iron is extracted by chloroform or carbon tetrachloride as the cupferron complex.

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THE EFFECT OF CYANIDE AND CARBON MONOXIDE ON THE OXYGEN CONSUMPTION OF *PARAMOECIUM CAUDATUM*

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The oxygen consumption of ciliates has often been claimed to be relatively unaffected by cyanide (Lund, 1918; Gerard and Hyman, 1931; Shoup and Boykin, 1931; Pitts, 1932). If correct, this would be of considerable theoretical interest, for the work of Keilin (1925, 1929, 1930) and others has demonstrated that in aerobic organisms generally, the greater part of the oxygen consumption is mediated by the cytochrome-cytochrome oxidase (CCO) system, a respiratory mechanism strongly inhibited by cyanide and carbon monoxide (Green, 1941). Recently, Hall (1941) has directed criticism at the earlier work on the effect of cyanide on the respiration of ciliates, suggesting that the failure to observe a considerable inhibition was due to imperfect technical methods. He draws attention to the necessity of using the balanced potassium hydroxide-potassium cyanide mixtures of Krebs (1935) and van Heyningen (1935) as the absorbent of respiratory carbon dioxide in place of the usual potash solution. If this precaution is not taken, loss of HCN vapour from the medium as a result of hydrolysis of the potassium cyanide may cause such a fall in the cyanide concentration that a respiratory inhibition is hardly to be expected. This gradual loss of hydrogen cyanide from the medium is prevented by using a KOH-KCN solution with a vapour pressure of HCN in equilibrium with that in the medium. A slow fall in the concentration of cyanide in the medium would provide an explanation for the observations of Pitts (1932) and Lwoff (1934) that the cyanide-inhibition of oxygen uptake in *Colpidium campylum* and *Glaucoma piriformis* respectively is only temporary. Hall finds that when this source of error has been eliminated, the oxygen consumption of *C. campylum* can be inhibited as much as 88 p.c. by M/1,000 potassium cyanide. He believes that a relative cyano-stability "characterizes the respiration neither of the protozoa generally nor of the ciliates in particular." It was the object of the present work to determine whether this conclusion is valid for *Paramoecium*, which has been the form most frequently examined in the past.

MATERIAL AND METHODS.

Hay infusion cultures of a pure line of *P. caudatum* were maintained in small tubes at room temperature. No attempt was made to culture the organisms aseptically in media of known chemical composition. The saline used for washing and resuspending the ciliates before an experiment was Tyrode solution diluted with forty times its volume of distilled water. Bicarbonate was omitted, a dilute phosphate buffer of final concentration M/150 being used to adjust the pH to 6·8. The organisms were freed from debris and most bacteria by centrifuging and washing in several portions of saline in the manner described by Boell and Woodruff (1941). We have confirmed their claim that serious errors due to bacterial respiration are eliminated if adequate care is taken during the washing process.

For the determination of the "normal" rate of oxygen consumption and its inhibition by cyanide, the Cartesian diver ultra-microrespirometer proved very satisfactory. In the construction and use of the apparatus, we have followed closely the descriptions given by Boell, Needham and Rogers (1939) and Boell and Woodruff (1941). Although Needham and his collaborators

have described a method for filling the divers with a gas phase of predetermined composition, we found it more convenient to use a Barcroft-Dixon respirometer (Dixon, 1934) for the experiments on the inhibitory action of carbon monoxide. This apparatus of course necessitated the use of dense suspensions of animals. Both respirometers were used in conjunction with water baths, all experiments being performed at 25° C. Following the procedure of Boell and Woodruff, results are expressed by the symbol Q'_{O_2}, the number of millimicrolitres of oxygen consumed per animal per hour. Volumes were corrected to N.T.P.

THE NORMAL OXYGEN UPTAKE.

The mean of ten experiments gave a value of 0·66 for the Q'_{O_2} of *P. caudatum* under the specified experimental conditions. The number of animals used in each diver ranged from 25 to 80. Typical figures are presented graphically in Fig. 1.

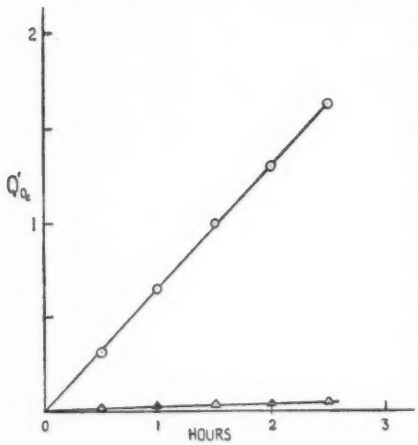

Fig. 1. Oxygen consumption of *P. caudatum* expressed in millimicrolitres per animal (Q'_{O_2}). Dilute saline medium (see text), pH 6·8, temperature 25° C. The experimental diver contained eighty animals. The lower curve (triangles) is for the control diver which contained saline medium but no protozoa.

The results obtained by several investigators are summarized in Table 1, indicating an extraordinary diversity of opinion as to the normal oxygen uptake. No doubt some of the differences are to be attributed to specific and individual variations in the size and physiological condition of the animals. Thus, Boell and Woodruff find the Q'_{O_2} of *P. calkinsi* to be 0·25 for animals with a positive mating tendency and 0·43 for animals showing no mating tendency. These authors calculate that if the highest figures claimed by earlier workers do actually represent the true rate of oxygen consumption, then all the organic material in a single paramoecium would be oxidized in from 7 to 18 hours. As paramoecia will survive much longer than this in the complete absence of food, it follows that these high values for the Q'_{O_2} must be in error. Cunningham and Kirk (1942) found the Q'_{O_2} to range from 1·50 to 5·35, the mean being 3·5. They believe that variation in cell volume will explain the large range of the Q'_{O_2} but admit that the absolute accuracy of their results is somewhat doubtful. It is interesting to note the good agreement between the results of those investigators who have used the Cartesian diver respirometer. This apparatus is undoubtedly the most satisfactory yet devised for the measurement of protozoan respiration, combining as it does extreme sensitivity with good stability. The small differences between the figures given by Boell and Woodruff for the Q'_{O_2} of *P. calkinsi* and *P. multimicronucleatum* and the figure given in the present paper for *P. caudatum* are probably due to size differences between the three species. Some preliminary experiments of our own indicate that variation in the pH between 6·2 and 7·4 has only a small effect on the Q'_{O_2}, the uptake being greater in the acid range. This is in general agreement with the observations of Hall (1941) on *C. campylum*.

TABLE 1.

Species.	Temperature.	Q'_{O_2}	Method.	Investigator.
P. caudatum	26	0·14	Winkler	Lund (1918)
,,	23	2·14	Winterstein	Zweibaum (1921)
,,	19	3·85	Warburg	Necheles (1924)
,,	21	5·2	capillary	Kalmus (1927)
,,	21	0·49	capillary	Howland and Bernstein (1931)
,,	—	3·5	capillary	Cunningham and Kirk (1942)
,,	25	0·66	Cartesian diver	Clark (this paper)
P. multimicronucleatum	25	1·00	Warburg	Mast *et al.* (1936)
,,	25	0·6	Cartesian diver	Boell and Woodruff (1941)
P. calkinsi ,,	25	0·45	Cartesian diver	Boell and Woodruff (1941)

THE EFFECT OF CYANIDE ON THE OXYGEN UPTAKE.

As the medium introduced into the diver already contained cyanide, it was not possible to compare the oxygen uptake of the same animals before and after the addition of the inhibitor. To overcome this difficulty, a separate diver was set up containing the same volumes of liquid and gas phases and approximately the same number of ciliates (to within two or three), but lacking the cyanide.

TABLE 2.

Per cent. inhibition of the Q'_{O_2} *by cyanide in the presence of glucose.*

Glucose content of medium.	M/1,000 KCN.	M/200 KCN.
0·002 p.c.	35 p.c.	45 p.c.
0·1 p.c.	50 p.c.	58 p.c.

The graph illustrated in Fig. 2 represents the mean results of five experiments with M/1,000 cyanide. The degree of inhibition is relatively low, about 35 p.c. Even in M/200 KCN, the inhibition was only about 45 p.c., but in both cases was quite constant over a period of at least five hours. Thus it seems highly probable that the temporary inhibition of oxygen uptake observed in certain ciliates by Lwoff (1934) and Pitts (1932) was due, as Hall suggests, to the gradual loss of hydrogen cyanide vapour from the medium.

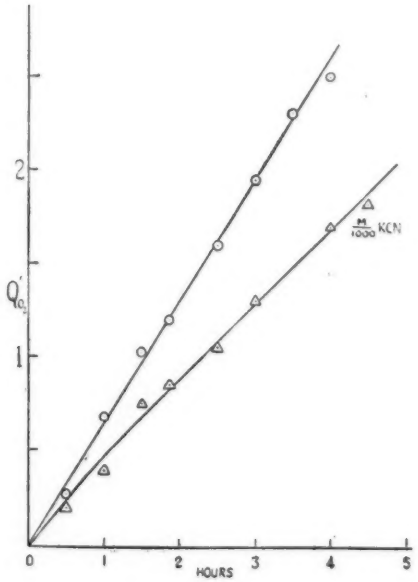

Fig. 2. The effect of M/1,000 potassium cyanide on the rate of oxygen consumption of *P. caudatum*, based on the mean results of five experiments. Respiratory carbon dioxide absorbed by a balanced KOH-KCN solution. The upper curve (circles) represent the oxygen uptake in the absence of cyanide, while the lower (triangles) that when cyanide is present.

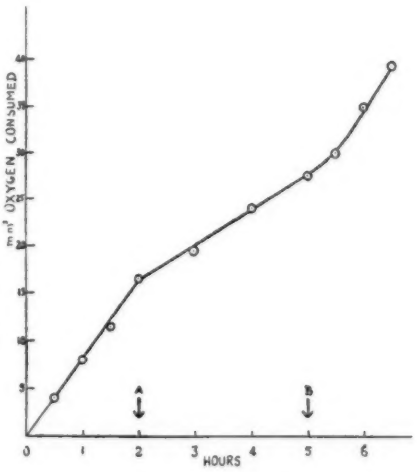

Fig. 3. The effect of carbon monoxide on the oxygen consumption of a dense suspension of *P. caudatum*. The original gas phase of 95 p.c. nitrogen and 5 p.c. oxygen replaced at time marked A by a mixture of 95 p.c. carbon monoxide and 5 p.c. oxygen. Respirometer in the dark from A to B. At B the entire apparatus was exposed to light.

The relative stability of the rate of oxygen consumption in the presence of cyanide and under the given conditions may indicate that the CCO system plays a smaller part in the aerobic respiration of *Paramoecium* than in metazoan cells generally (and in certain Protista); or it might provide yet a further example of an apparent cyano-stability which in reality is due to an incomplete saturation of the CCO system with substrate. If saturation is incomplete, the functioning of the respiratory system will obviously not be maximal (Commoner, 1939). To elucidate this point, the assumption was made that the principle substrate oxidized in *Paramoecium* is carbohydrate (see also Hall). If this holds, then increasing the glucose content of the medium should give complete saturation of the CCO system, resulting in an increased cyano-sensitivity. This expectation was partly realized experimentally, the data being summarized in Table 2. However, the maximum inhibition obtained with M/200 cyanide was still only 58 p.c. Thus incomplete saturation of the CCO system with substrate may be a partial explanation of the reported lack of cyano-sensitivity in *Paramoecium*, but it is not a complete explanation. This is the more interesting in view of Hall's observations that the inhibition of the Q'_{O_2} of *C. campylum* is almost maximal in M/1,000 KCN and averages 88 p.c., provided adequate carbohydrate is present in the medium. It would appear that even between ciliates as closely related as *Colpidium* and *Paramoecium*, there may be considerable differences in the chief respiratory mechanisms and, perhaps, in the nature of the principle substrate utilized.

THE EFFECT OF CARBON MONOXIDE ON THE OXYGEN CONSUMPTION.

As an inhibitor of cytochrome oxidase activity, carbon monoxide is even more specific than cyanide, provided that it is present at high partial pressure and in the dark. The carbon monoxide-cytochrome oxidase compound dissociates when exposed to light, liberating the active oxidase. Thus a light-reversible inhibition of oxygen uptake by carbon monoxide can be taken as good evidence for the active presence of the CCO system (Green, 1941).

To enable a rapid change to be made in the nature of the gas phase to which the organisms were exposed, the Barcroft-Dixon respirometer was used. The paramoecia were washed and resuspended in the usual manner, the final suspension containing from 12,000 to 16,000 animals. It was not necessary to determine the exact number of animals present, for a preliminary experiment in the absence of any carbon monoxide served as a measure of the "normal" rate of oxygen consumption of the whole suspension, with which figure the inhibited uptake could be compared. During the preliminary period, which lasted from one to two hours, the gas phase present in the chambers consisted of 95 p.c. nitrogen and 5 p.c. oxygen (by volume). At the desired moment, the chambers were washed out with a slow stream of the carbon monoxide-oxygen mixture (95: 5) for ten minutes. The taps were then closed and the entire respirometer kept in darkness for about three hours, readings being made every 30 minutes. Before the conclusion of each experiment, the apparatus was exposed to a bright light, the gas phase remaining unchanged, and readings were continued for a further 90 minutes. This latter period of the experiment aimed at demonstrating the light-reversibility of any inhibition that might be observed. In every case, the medium contained sufficient carbohydrate to ensure complete saturation of the CCO system, should it be present.

In five experiments, the inhibition ranged from 50 p.c. to 60 p.c., the mean value of the partition constant, as calculated from the Warburg-Negelein equation, being 15. In every case, the rate of oxygen uptake returned to approximately its original value when the respirometer was illuminated (see Fig. 3).

DISCUSSION.

As both cyanide and carbon monoxide exert an inhibitory action on the oxygen consumption of *Paramoecium*, it can be concluded that the CCO system is present in this protozoan. The presence of cytochrome oxidase was indicated by the experiments of Child and Deviney (1926) on the indophenol blue reaction, but the reports of a cyano-stability of aerobic respiration have presented stumbling blocks to any attempt to homologize protozoan respiratory mechanisms with those of most aerobic Metazoa. That the CCO system does play a major role in the respiration of some ciliates at least is indicated by the work of Hall on *C. campylum*, which has been confirmed in part in this laboratory. Furthermore, direct spectroscopic examination of a dense suspension of this ciliate reveals the bands of cytochrome c (Clark, unpublished experiments). On the other hand, the examination of a dense suspension of *P. caudatum* shows only a faint band at 550 mμ. After treatment with sodium hydrosulphite and pyridine, both *Colpidium* and *Paramoecium* show the bands of pyridine-haemochromogen. Lwoff (1933) has

also obtained spectroscopic evidence which supports the contention that the CCO system functions in the Protozoa.

The relative stability of about 60 p.c. of the oxygen uptake of *Paramoecium* in the presence of inhibitors of cytochrome oxidase indicates the presence of an alternative respiratory pathway, perhaps a flavoprotein system. As Jahn (1941) points out, it does seem possible that more than one respiratory system might be present in organisms which, although primarily aerobic, are nevertheless often exposed to nearly anaerobic conditions. It is already known that a flavoprotein mechanism is of considerable importance for the respiration of anaerobes generally. In the presence of an inhibitor of cytochrome oxidase, such a flavoprotein mechanism might be capable of mediating rather more than half of the "normal" respiration of *Paramoecium*. It would be of considerable interest to determine whether the oxidation-reduction reactions known to be carried out by flavoproteins, in the presence of co-enzyme I, occur in protozoans. Ball (1942) has suggested that even in the presence of an inhibitor such as cyanide, the oxidation-reduction potential of cytochrome oxidase is sufficiently high to enable it to react with substances of very low potential, such as flavoprotein, but not with any of the three components of cytochrome, which all have high potentials. He offers this as an explanation of the fact that although cytochrome oxidase is present in the unfertilized egg of *Arbacia* (Krahl *et al.*, 1941), the oxygen consumption is not cyanide sensitive.

Dr. Libbie Hyman of the American Museum of Natural History has kindly placed at our disposal the results of some unpublished experiments in which she found that ".... cyanide (M/250 to M/1,000) depresses the oxygen consumption of non-starved *Paramoecium* to a maximum of 35 p.c. but has no effect on starved ones." As Lund (1918) found the oxygen consumption of *Paramoecium* to increase markedly after feeding, it would appear that, as Hyman suggests, the extra oxygen consumption following feeding is cyanide-sensitive, while the "basal" respiration is not.

Whether the CCO system is the main respiratory pathway in the Protozoa or not, the present work indicates that our experimental facts are still too incomplete to enable generalizations to be made. It is certainly striking that, within the ciliates at least, two closely related forms can show what are presumably marked differences in their respiratory mechanisms.

Lwoff (1933) has found the respiration of several flagellates to be cyanide sensitive, while Clark (1942) states that about 70 p.c. of the oxygen consumption of *Amoeba proteus* is inhibited by potassium cyanide. Recently, Pace and Belda (1944) have reported that the respiration of *Pelomyxa carolinensis* can be reduced by 63 p.c. with M/100,000 KCN. Thus the cyano-stability of the respiration of *Paramoecium* may well prove to be unique among the Protozoa. It is intended that further work along these lines should be directed primarily to the question of whether, as Hall suggests, carbohydrate is the principle substrate oxidized by the Protozoa, or whether the substrate is predominantly carbohydrate in an organism such as *Colpidium*, but chiefly protein in a relatively cyanide resistant form such as *Paramoecium*.

SUMMARY.

The mean rate of oxygen consumption of *Paramoecium caudatum* was found to be 0·66 millimicrolitres per animal per hour at 25° C. and pH 6·8.

In the presence of carbohydrate, M/200 potassium cyanide inhibits the oxygen uptake by about 58 p.c.

Carbon monoxide at high partial pressures inhibits the oxygen uptake by 50 p.c. The inhibition is light reversible.

About half of the aerobic respiration in *P. caudatum* appears to be mediated by the cytochrome-cytochrome oxidase system.

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THE EFFECT OF GLYCINE ON THE AGGLUTINATION OF HUMAN ERYTHROCYTES

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Glycine has been used in America to counteract the rouleaux formation caused by gelatin. Patients who have received recent gelatin transfusions show extreme rouleaux formation when cross-matching is done before subsequent blood transfusion.

Vogelaar (1943) suggested that glycine might be expected to be absorbed readily on the surface of erythrocytes and so exert some effect on this rouleaux formation. He found that rouleaux were not formed when erythrocytes were suspended in 5 p.c. gelatin in the presence of 0·28 M. glycine (2·1 p.c.).

This was confirmed by Hughes (1944) who said that 0·3 M. glycine (2·25 p.c.) was hypertonic and produced "warping" of the red cells sufficient to interfere with rouleaux formation. Koop and Bullitt (1944) stated that 1 p.c. glycine in physiological saline in no way interfered with the grouping or cross-matching of blood.

A series of studies has been made on the effect of glycine on human erythrocytes and on their agglutination by isohaemagglutinins.

TECHNIQUE.

For slide agglutination flat glass plates were used, painted white on the under-surface and divided into compartments by painted lines. One drop of serum was added to one drop of cell suspension and the plate rock-rotated continuously. When glycine was present in the red cell suspension its final concentration on the slide was thus one-half of the original concentration.

Tube agglutination was done as described by Weiner (1943) in test tubes 3" × $\frac{5}{8}$" (WR). Agglutinating serum (0·2 ml.) was diluted serially with the test solution. A 1 p.c. suspension of red cells in the test solution was then added in equal amounts and results read after two hours at room temperature with occasional shaking.

RESULTS.

Suspension of Erythrocytes in Glycine Solutions.

A few drops of defibrinated blood were added to 10 ml. amounts of various aqueous solutions of glycine:

Glycine.	Agglutination.	Haemolysis.
1 p.c.	+	++
2 p.c.	+	+
4 p.c.	+++	−
8 p.c.	++	− eventually +
16 p.c.	+	−

It was found that some of the cells haemolysed fairly rapidly in concentrations of 2 p.c. and less, and that suspensions in solutions of higher concentrations showed a small amount of haemolysis after some hours. All suspensions showed spontaneous clumping of the red cells within a few minutes.

Microscopic examination of the clumped cells showed an appearance quite resembling red cells agglutinated by an agglutinating serum. There were no associated rouleaux as are seen in cells clumped by gelatin.

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Dispersion of Glycine-Agglutinated Red Cells.

The following observations were made:

(a) Vigorous shaking of cells clumped in glycine solutions, and compression between coverslip and slide did not disperse the clumps.

(b) If glycine-clumped cells were centrifuged and resuspended in isotonic saline or sodium citrate they dispersed readily on shaking.

(c) The addition of a small quantity of concentrated sodium chloride or sodium citrate solution to cells clumped in 4 p.c. glycine dispersed the clumps. This may be watched under the microscope if a small drop of 20 p.c. sodium chloride solution is placed at the edge of the coverslip.

(d) Cells suspended in solutions of isotonic sodium chloride or sodium citrate with varying concentrations of glycine (0·5–16 p.c.) were not clumped or haemolysed.

Effect of Glycine on the Slide Agglutination of Red Cells.

Suspensions of cells in physiological saline containing either no glycine or varying amounts thereof were added to agglutinating serum on a slide, and the results compared.

It was found that cells in suspensions containing 4–8 p.c. glycine showed agglutination in a much shorter time than those suspended in physiological saline. This effect was seen with all but two sera when 186 different sera (O, A and B) were allowed to agglutinate various A, B and AB cells suspended in 4 p.c. glycine in physiological saline. The two sera with which no acceleration of agglutination was seen were pooled typing sera of titre 1/512 and were avid, causing rapid agglutination of cells in physiological saline alone. When diluted 1/4 with serum containing no agglutinin (AB serum) these sera showed acceleration of agglutination by glycine.

Suspensions of cells from 35 different donors (A and B) in 4 p.c. glycine in physiological saline were all agglutinated more rapidly than when suspended in physiological saline alone. A2 cells suspended in physiological saline, and in physiological saline containing 4 p.c. glycine, were agglutinated with B serum (titre 1/8 against cells used) and the times noted for the first appearance of agglutination.

Physiological saline: Mean 113 seconds. Standard deviation 15·3 seconds.

4 p.c. glycine-saline: Mean 49 seconds. Standard deviation 5·8 seconds.

Difference: 64 seconds.

Standard deviation of difference: 16·36 seconds.

$$\frac{\text{Difference}}{\text{Standard deviation of difference}} : 3{\cdot}912.$$

That is, with this serum-cell combination, agglutination took place in less than one-half the time when 4 p.c. glycine was present in the red cell suspension, and the probability of this being a chance occurrence was less than 1 in 10,000.

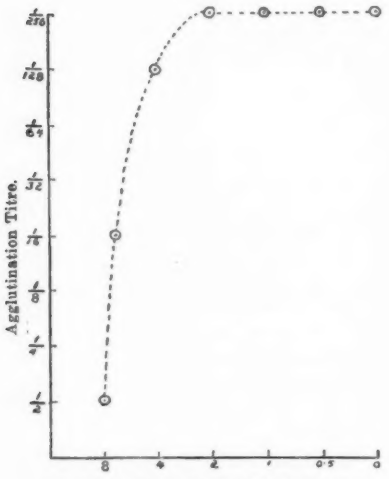

Fig. 1.

Glycine thus caused an acceleration of the time for first appearance of agglutination. It was further noticed that larger clumps formed more rapidly in the presence of glycine although the ultimate appearance of the agglutinated cells on the slide was much the same.

The presence of glycine did not affect the specificity of the agglutination reaction.

Effect of Glycine on the Titration of Agglutinating Serum.

Cells suspended in physiological saline containing different concentrations of glycine were acted on by agglutinating sera diluted with like solutions of glycine in saline, and the titre plotted at which agglutination occurred in each series. (Fig 1).

The presence of glycine up to 2–4 p.c. did not cause any appreciable change in the serum concentration at which the cells were agglutinated, but with concentrations above this range there was increasing inhibition of agglutination.

The acceleration of agglutination by glycine may be seen in tube agglutination.

Inhibition of Agglutination by High Concentration of Glycine.

Using serum with titre 1/256, cells were suspended in saline containing 16 p.c. glycine,

and agglutinating serum (final dilution of 1 in 8) was added as for tube agglutination. No agglutination occurred. The cells were centrifuged and re-suspended in the supernatant. Still there was no agglutination. They were again centrifuged and re-suspended in physiological saline. Agglutination took place on standing, or on centrifuging and shaking up again in the supernatant. Agglutination occurred also if these cells, exposed to agglutinating serum in the presence of high concentration of glycine, were washed with the glycine-saline solution and then re-suspended in physiological saline.

Further, red cells suspended in 16 p.c. glycine in physiological saline were acted upon by serum diluted serially with the same solution, then centrifuged and re-suspended in physiological saline. Agglutination took place with the same serum dilution as in a control titration using physiological saline alone.

Thus a high concentration of glycine does not prevent the agglutinin from combining with the red cells, and this union is as complete as occurs in physiological saline suspensions if subsequent agglutination of the cells in a suitable suspending medium may be taken as a criterion.

DISCUSSION.

These studies have shown that:

(a) Erythrocytes are agglutinated by glycine: but this does not occur when 0·85 p.c. sodium chloride or 2·6 p.c. sodium citrate is present.

(b) The agglutination of erythrocytes by agglutinating serum on slide or in tube is accelerated by glycine in optimum concentrations.

(c) High concentrations of glycine inhibit the agglutination of erythrocytes by agglutinating serum but do not prevent the combination of agglutinin with agglutinogen.

When red cells clumped by glycine are observed under the microscope they cannot be distinguished from cells agglutinated by agglutinating serum.

A suspension of red cells probably remains as such by virtue of the electric charge on the surface of the cells. Tannin agglutinates erythrocytes probably by some physico-chemical modification of the cell surface (Boyd, 1943). It is considered that glycine may act in a similar manner, having available amino and carboxyl radicles for combination with molecules on the surface of the red cell. Salts, such as sodium chloride and sodium citrate, may prevent or disrupt this combination and so prevent the agglutination of cells by glycine, or disperse them if already agglutinated. In view of the possibility of a chemical combination of glycine with the red cell it is interesting to note that Beach *et al.* (1939) discussing the amino acid content of the post-haemolytic residue of red cells, make no mention of glycine as one of the constituents.

Why glycine should disperse the rouleaux caused by gelatin remains obscure. These rouleaux can also be partially dispersed by 5 p.c. glucose. Until the whole mechanism of the agglutination of red cells is known, and the structure, composition, and chemical combining powers of the red cell itself elucidated, discussion of these confusing observations must remain speculative.

Two stages are commonly distinguishable in serological reactions. In the first occurs the specific union of antigen and antibody. In the second the suspension of sensitized particles bearing the antigen undergoes a visible change such as agglutination. The first stage is probably completed in a very short time and occurs in the absence, or excess of electrolytes. The acceleration of red cell agglutination by an agglutinating serum in the presence of glycine appears to be due to some modification of the second stage of the process.

The mechanism of the second stage is complex. Probably several factors take part, such as chemical combination, alteration in the surface charge on the red cell and changes in interfacial tension. How glycine exerts its action is uncertain. Aqueous solutions of glycine are essentially neutral. Its ionic concentration in the suspending medium in relation to the ions of sodium chloride is possibly different from that at the surface of the red cell (Danielli, 1944). An increase in the concentration of the salts commonly used for suspending red cells

—sodium chloride and sodium citrate—also results in acceleration of agglutination by agglutinating serum (sodium chloride—Sachs (1942), sodium citrate—personal observation). Also very small quantities of electrolytes when added to salt-free suspensions of erythrocytes (e.g. in sugar) will cause agglutination.

Whether the acceleration of agglutination by glycine is comparable to that caused by increased electrolyte concentration or actual chemical combination with the red cell cannot be stated definitely. An attractive explanation is that the combination of agglutinin with the red cell allows glycine to exert its agglutinating effect which had previously been kept in abeyance by the sodium chloride or citrate present—thus accelerating the second stage of agglutination.

The inhibition of agglutination by higher concentrations of glycine again resembles the effect caused by salts such as sodium chloride, citrate and sulphate, calcium chloride, ammonium sulphate and potassium iodide (personal observations). But non-electrolytes such as glucose in physiological saline will also inhibit agglutination. With glucose this effect is seen in concentrations over 4 p.c. (personal observation). The manner in which these solutes prevent the clumping of sensitized red cells is uncertain. It is probably due to changes at the interface between cell and suspending medium. Certainly the high concentration of solute does not prevent the combination of agglutinin with agglutinogen.

Wright and MacCallum (1922), using a haemolytic system, confirmed the observations of earlier workers by demonstrating that antigen combined with antibody in hypertonic saline solutions. They showed that haemolysis did not occur and concluded that the action of the increased electrolyte content was upon complement—preventing it from effecting lysis of sensitized cells. Topley and Wilson (1936) state that this is due to inhibition of the union of complement with sensitized red cells. Here, then, the block in the system is due to prevention of union of red cell with complement—part of which, at least, is of protein nature. In the inhibition of agglutination sensitized red cells are prevented from adhering to each other. The manner in which glucose produces this result is obscure, and it would appear that the explanation is a physical one—a change in the physical properties of the antigen-antibody complex on the surface of the red cells. That is the sensitized red cells have more attraction for the surrounding medium than they have for each other when there is a high solute content in that medium.

The physiological function of human isohaemagglutinins and agglutinogens has not yet been explained. It has been usual to demonstrate their presence using cells suspended in isotonic saline or sodium citrate. The acceleration of the reaction by various solutes is of practical importance in cross-matching prior to transfusion, especially when dealing with recipient's serum of low titre or with A_2 donor's cells. This only applies to freshly collected samples of donor's cells, as erythrocytes are less readily agglutinated after storage in hypertonic solutions than in isotonic solutions.

SUMMARY.

Red cells are agglutinated when suspended in isotonic and hypertonic solutions of glycine.

This agglutination does not occur in the presence of 0·85 p.c. sodium chloride or 2·6 p.c. sodium citrate.

Cells agglutinated by glycine can be dispersed by the addition of sodium chloride.

Slide agglutination of red blood cells by agglutinating serum is accelerated by the presence of 4 p.c. glycine.

Higher concentrations of glycine inhibit the agglutination of red cells by agglutinating serum but do not prevent the combination of agglutinin with agglutinogen.

The mode of action of glycine is discussed and compared with the effects of various salts and glucose.

The practical application of accelerated agglutination is indicated.

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